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(54) Title: NUCLEIC ACID LIGANDS THAT BIND TO AND INHIBIT DNA POLYMERASES			
(57) Abstract This invention discloses high-affinity oligonucleotide ligands to the thermostable <i>Taq</i> polymerase and <i>Tth</i> polymerase. Specifically, this invention discloses DNA ligands having the ability to bind to the <i>Taq</i> and <i>Tth</i> polymerases and the methods for obtaining such ligands. The ligands are capable of inhibiting polymerases at ambient temperatures.			

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NUCLEIC ACID LIGANDS THAT BIND TO AND INHIBIT DNA POLYMERASES**FIELD OF THE INVENTION**

5 Described herein are methods for identifying and preparing high-affinity nucleic acid ligands to DNA polymerases, specifically thermostable DNA polymerases. In a preferred embodiment the DNA polymerase is selected from *Taq* polymerase, a thermostable polymerase isolated from *Thermus aquaticus* or *Tth* polymerase, a thermostable DNA polymerase and
10 reverse transcriptase isolated from *Thermus thermophilus*. However, the method of this invention can be extended to the identification and preparation of any thermal stable DNA polymerase. The method utilized herein for identifying such nucleic acid ligands is called SELEX, an acronym for Systematic Evolution of Ligands by EXponential Enrichment. Also described
15 herein is an improved method for performing the Polymerase Chain Reaction using the nucleic acid ligands of this invention. Specifically disclosed herein are high-affinity nucleic acid ligands to *Taq* polymerase and *Tth* polymerase. The invention includes high-affinity DNA ligands which bind to *Taq* polymerase and *Tth* polymerase, thereby inhibiting their ability to polymerase
20 DNA synthesis at ambient temperatures. Further included within this invention are nucleic acid switches. The thermal dependent binding of the nucleic acid ligands to DNA polymerases of this invention are examples of ligands whose desirable properties can be switched on or off based on any number of reaction conditions.

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BACKGROUND OF THE INVENTION

The Polymerase Chain Reaction (PCR), is a recently developed technique which has had a significant impact in many areas of science. PCR is a rapid and simple method for specifically amplifying a target DNA sequence

in an exponential manner. (Saiki *et al.* (1985) *Science* 230:1350; Mullis and Faloona (1987) *Methods Enzymol.* 155:335). Briefly, the method consists of synthesizing a set of primers that have nucleotide sequences complementary to the DNA that flanks the target sequence. The primers are then mixed with a
5 solution of the target DNA, a thermostable DNA polymerase and all four deoxynucleotides (A, T, C and G). The solution is then heated to a temperature sufficient to separate the complementary strands of DNA (approximately 95°C) and then cooled to a temperature sufficient to allow the primers to bind to the flanking sequences. The reaction mixture is then heated
10 again (to approximately 72°C) to allow the DNA synthesis to proceed. After a short period of time the temperature of the reaction mixture is once again raised to a temperature sufficient to separate the newly formed double-stranded DNA, thus completing the first cycle of PCR. The reaction mixture is then cooled and the cycle is repeated. Thus, PCR consists of repetitive cycles of
15 DNA melting, annealing and synthesis. Twenty replication cycles can yield up to a million fold amplification of the target DNA sequence. The ability to amplify a single DNA molecule by PCR has applications in environmental and food microbiology (Wernars *et al.* (1991) *Appl. Env. Microbiol.* 57:1914-1919; Hill and Keasler (1991) *Int. J. Food Microbiol.* 12:67-75),
20 clinical microbiology (Wages *et al.* (1991) *J. Med. Virol.* 33:58-63; Sacramento *et al.* (1991) *Mol. Cell Probes* 5:229-240; Laure *et al.* (1988) *Lancet* 2:538), oncology (Kumar and Barbacid (1988) *Oncogene* 3:647-651; McCormick (1989) *Cancer Cells* 1:56-61; Crescenzi *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:4869), genetic disease prognosis (Handyside *et al.* (1990) *Nature* 344:768-770), blood banking (Jackson (1990) *Transfusion* 30:51-57) and forensics (Higuchi *et al.* (1988) *Nature (London)* 332:543).

The availability of thermostable DNA polymerases such as *Taq* DNA polymerase has both simplified and improved PCR. Originally only heat-sensitive polymerases, such as *E. coli* DNA polymerase were available for use

in PCR. Heat-sensitive polymerases, however, are destroyed at the temperatures required to melt double-stranded DNA, and additional polymerase has to be added after each PCR cycle. *Taq* DNA polymerase, isolated from the thermophilic bacterium *Thermus aquaticus*, is stable up to 95°C and its use in PCR has eliminated the necessity of repetitive addition of temperature sensitive polymerases after each thermal cycle. Additionally, because *Taq* polymerase can be used at higher temperatures it has improved the specificity and sensitivity of PCR. The reason for the improved specificity is that at higher temperatures the binding of promoters to sites other than the desired ones (referred to as mispriming) is significantly reduced.

Since its discovery, the Polymerase Chain Reaction has been modified for various applications, such as *in situ* PCR, in which the detection limit of traditional *in situ* hybridization has been pushed to the single copy level (Haase *et al.* (1990) Proc. Natl. Acad. Sci. USA 87:4971-4975), and reverse transcriptase PCR (RT-PCR), wherein an RNA sequence is converted to its copy DNA (cDNA) by reverse transcriptase (RT) before being amplified by PCR, making RNA a substrate for PCR (Kawasaki (1991) Amplification of RNA in PCR Protocols, A Guide to Methods and Applications, Innis *et al.*, Eds. Academic Press Inc., San Diego, CA, 21-27). Mesophilic viral reverse transcriptases, however, are often unable to synthesize full-length cDNA molecules because they cannot "read through" stable secondary structures of RNA molecules. This limitation has recently been overcome by use of a polymerase isolated from *Thermus thermophilus* (*Tth* polymerase). *Tth* polymerase is a thermostable polymerase that can function as both reverse transcriptase and DNA polymerase (Myers and Gelfand (1991) Biochemistry 30:7662-7666). The reverse transcription performed at an elevated temperature using *Tth* polymerase eliminates secondary structures of template RNA, making it possible for the synthesis of full-length cDNA.

Although significant progress has been made in PCR technology, the

amplification of nontarget oligonucleotides due to side-reactions, such as mispriming of background DNA and/or primer oligomerization still presents a significant problem. This is especially true in diagnostic applications in which PCR is carried out in a milieu containing background DNA while the target DNA may be present in a single copy (Chou *et al.* (1992) *Nucleic Acid Res.* 20:1717-1723). It has been determined that these side reactions often occur when all reactants have been mixed at ambient temperature before thermal cycling is initiated.

Two methods have been reported which minimize these side reactions. In the first method, termed "hot start" PCR (Chou *et al.* (1992) *Nucleic Acid Res.* 20:1717-1723; D'Aquila *et al.* (1991) *Nucleic Acid Res.* 19:3749), all of the reagents are heated to 72°C before adding the final reagent, usually the polymerase. In wax-mediated "hot start" PCR, a component(s) crucial to polymerase activity is physically separated from the rest of the reaction mixture at low temperature by a wax layer which melts upon heating in the first cycle (Chou *et al.* (1992) *Nucleic Acids Res.* 20:1717; Horton *et al.* (1994) *BioTechniques* 16:42). "Hot start" PCR has certain drawbacks; the requirement of reopening of tubes before initiating thermocycling increases crossover contamination and repetitive pipetting makes it tedious in handling a large number of samples. A reagent that could be placed directly in the reaction mixture with all other reaction components and inhibit the polymerase at ambient temperature would be useful to overcome limitations associated with "hot start" PCR. Although this method does increase specificity, thereby reducing side products, the method is inconvenient for dealing with a large number of samples, the reaction mixture can become more easily contaminated, and the method is error-prone.

In the second method, a neutralizing antibody to *Taq* polymerase (referred to as *TaqStart*) is added to the complete reaction mixture. This antibody inhibits the polymerase activity at ambient temperature (Kellogg *et*

al. (1994) *BioTechniques* 16:1134-1137), but is inactivated by heat denaturation once the reaction is thermocycled, rendering the polymerase active. The drawback of this approach to reducing side products is that the anti-*Taq* antibody should be stored at -20°C until use, which means that
5 detection kits should be packaged and shipped under controlled environment adding to their cost. In addition, a significant amount of antibody (~ 1 µg of antibody/5 U of *Taq* polymerase) is needed for a single PCR.

The development of high affinity nucleic acid ligands capable of inhibiting the thermostable *Taq* and *Tth* polymerases would obviate the need
10 for the "hot start" method and would overcome the limitations associated with the second method. Nucleic acid inhibitors can be developed that are extremely specific and have high affinity. Since nucleic acids are more stable than proteins at ambient temperature, the shipping and packaging problems associated with using antibodies can be overcome. Additionally, nucleic acids,
15 like antibodies can be identified that will lose their affinity for the polymerase at higher temperatures, allowing the polymerase to be activated when desired. The potential for mispriming mediated by nucleic acid based inhibitors themselves functioning as primers (in addition to the specific primers used in the reaction) in PCR can be eliminated by capping their 3' ends.

20 X-ray crystal structures of several DNA polymerases have indicated that they fold into similar three dimensional structures. (For a review, see Joyce and Steitz (1994) *Annu. Rev. Biochem.* 63:777). The C-terminal domain responsible for polymerization is organized into three sub-domains representing "palm", "fingers", and "thumb", anatomically analogous to a right
25 hand. *Tth* polymerase and *Taq* polymerase are 93% similar and 88% identical at the amino acid sequence level (Abramson (1995) in PCR Strategies (Academic Press, New York). Both are devoid of 3'→5' exonuclease activity, but contain 5'→3' exonuclease activity (Abramson (1995) in PCR Strategies (Academic Press, New York); Tindall and Kunkel (1988) *Biochemistry*

27:6008). Thus, nucleic acid ligand inhibitors might be expected to behave similarly toward both of these enzymes, as well as, other thermostable polymerases. This would make possible the use of a single inhibitor for a number of thermostable enzymes.

5

SELEX

A method for the *in vitro* evolution of nucleic acid molecules with highly specific binding to target molecules has been developed. This method, Systematic Evolution of Ligands by EXponential enrichment, termed SELEX, is described in United States Patent Application Serial No. 07/536,428, entitled "Systematic Evolution of Ligands by Exponential Enrichment," now abandoned, United States Patent Application Serial No. 07/714,131, filed June 10, 1991, entitled "Nucleic Acid Ligands," now United States Patent No. 5,475,096, United States Patent Application Serial No. 07/931,473, filed August 17, 1992, entitled "Nucleic Acid Ligands," now United States Patent No. 5,270,163 (see also PCT/US91/04078), each of which is herein specifically incorporated by reference. Each of these applications, collectively referred to herein as the SELEX Patent Applications, describes a fundamentally novel method for making a nucleic acid ligand to any desired target molecule.

The SELEX method involves selection from a mixture of candidate oligonucleotides and step-wise iterations of binding, partitioning and amplification, using the same general selection scheme, to achieve virtually any desired criterion of binding affinity and selectivity. Starting from a mixture of nucleic acids, preferably comprising a segment of randomized sequence, the SELEX method includes steps of contacting the mixture with the target under conditions favorable for binding, partitioning unbound nucleic acids from those nucleic acids which have bound specifically to target molecules, dissociating the nucleic acid-target complexes, amplifying the

nucleic acids dissociated from the nucleic acid-target complexes to yield a ligand-enriched mixture of nucleic acids, then reiterating the steps of binding, partitioning, dissociating and amplifying through as many cycles as desired to yield highly specific, high affinity nucleic acid ligands to the target molecule.

- 5 The basic SELEX method has been modified to achieve a number of specific objectives. For example, United States Patent Application Serial No. 07/960,093, filed October 14, 1992, entitled "Method for Selecting Nucleic Acids on the Basis of Structure," describes the use of SELEX in conjunction with gel electrophoresis to select nucleic acid molecules with specific
- 10 structural characteristics, such as bent DNA. United States Patent Application Serial No. 08/123,935, filed September 17, 1993, entitled "Photoselection of Nucleic Acid Ligands" describes a SELEX based method for selecting nucleic acid ligands containing photoreactive groups capable of binding and/or photocrosslinking to and/or photoinactivating a target molecule. United States
- 15 Patent Application Serial No. 08/134,028, filed October 7, 1993, entitled "High-Affinity Nucleic Acid Ligands That Discriminate Between Theophylline and Caffeine," describes a method for identifying highly specific nucleic acid ligands able to discriminate between closely related molecules, termed Counter-SELEX. United States Patent Application Serial No.
- 20 08/143,564, filed October 25, 1993, entitled "Systematic Evolution of Ligands by EXponential Enrichment: Solution SELEX," describes a SELEX-based method which achieves highly efficient partitioning between oligonucleotides having high and low affinity for a target molecule. United States Patent Application Serial No. 07/964,624, filed October 21, 1992, entitled "Methods
- 25 of Producing Nucleic Acid Ligands," now United States Patent No. 4,496,938, describes methods for obtaining improved nucleic acid ligands after SELEX has been performed. United States Patent Application Serial No. 08/400,440, filed March 8, 1995, entitled "Systematic Evolution of Ligands by EXponential Enrichment: Chemi-SELEX," describes methods for covalently

linking a ligand to its target.

The SELEX method encompasses the identification of high-affinity nucleic acid ligands containing modified nucleotides conferring improved characteristics on the ligand, such as improved *in vivo* stability or improved delivery characteristics. Examples of such modifications include chemical substitutions at the ribose and/or phosphate and/or base positions.

SELEX-identified nucleic acid ligands containing modified nucleotides are described in United States Patent Application Serial No. 08/117,991, filed September 8, 1993, entitled "High Affinity Nucleic Acid Ligands Containing Modified Nucleotides," that describes oligonucleotides containing nucleotide derivatives chemically modified at the 5- and 2'-positions of pyrimidines.

United States Patent Application Serial No. 08/134,028, *supra*, describes highly specific nucleic acid ligands containing one or more nucleotides modified with 2'-amino (2'-NH₂), 2'-fluoro (2'-F), and/or 2'-O-methyl (2'-OMe). United States Patent Application Serial No. 08/264,029, filed June 22, 1994, entitled "Novel Method of Preparation of 2' Modified Pyrimidine Intramolecular Nucleophilic Displacement," describes oligonucleotides containing various 2'-modified pyrimidines.

The SELEX method encompasses combining selected oligonucleotides with other selected oligonucleotides and non-oligonucleotide functional units as described in United States Patent Application Serial No. 08/284,063, filed August 2, 1994, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Chimeric SELEX" and United States Patent Application Serial No. 08/234,997, filed April 28, 1994, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Blended SELEX," respectively. These applications allow the combination of the broad array of shapes and other properties, and the efficient amplification and replication properties, of oligonucleotides with the desirable properties of other molecules. Each of the above described patent applications which describe modifications of the basic

SELEX procedure are specifically incorporated by reference herein in their entirety.

BRIEF SUMMARY OF THE INVENTION

5 The present invention includes methods of identifying and producing nucleic acid ligands to DNA polymerases. Specifically included are methods for identifying nucleic acid ligands to thermostable DNA polymerases useful in the Polymerase Chain Reaction, including the *Taq* and *Tth* polymerases and the nucleic acid ligands so identified and produced. More particularly, DNA
10 sequences are provided that are capable of binding specifically to the *Taq* and *Tth* polymerases respectively, thereby inhibiting their ability to catalyze the synthesis of DNA at ambient temperatures. The method of this invention can be extended to identifying and producing nucleic acid ligands to any thermostable DNA polymerase and the ligands so identified and produced.

15 Further included in this invention is a method of identifying nucleic acid ligands and nucleic acid ligand sequences to the *Taq* and *Tth* polymerases comprising the steps of (a) preparing a candidate mixture of nucleic acids, (b) partitioning between members of said candidate mixture on the basis of affinity to the *Taq* or *Tth* polymerases and (c) amplifying the selected
20 molecules to yield a mixture of nucleic acids enriched for nucleic acid sequences with a relatively higher affinity for binding to the *Taq* and *Tth* polymerases, respectively.

 Further included in this invention is an improved method of performing the Polymerase Chain Reaction comprising the step of including a nucleic acid
25 ligand that inhibits the thermostable polymerase at ambient temperatures, but dissociates from the polymerase at elevated temperatures. Such nucleic acid ligands are identified according to the method of this invention.

 More specifically, the present invention includes the ssDNA ligands to *Taq* polymerase and *Tth* polymerase identified according to the above-

described method, including those ligands listed in Tables 2 - 5 (SEQ ID NOS:7-73). Also included are DNA ligands to *Taq* polymerase and *Tth* polymerase that are substantially homologous to any of the given ligands and that have substantially the same ability to bind and inhibit the activity of *Taq* polymerase and *Tth* polymerase. Further included in this invention are DNA ligands to *Taq* polymerase and *Tth* polymerase that have substantially the same structural form as the ligands presented herein and that have substantially the same ability to bind and inhibit the activity of *Taq* polymerase and *Tth* polymerase.

The present invention also includes modified nucleotide sequences based on the DNA ligands identified herein and mixtures of the same.

The nucleic acid ligands of the present invention may function as "switches" in that they turn the Polymerase Chain Reaction "on" or "off" depending on the temperature of the reaction mixture. The present invention, therefore, also includes a method for identifying and preparing nucleic acid ligand sequences which function as switches comprising the steps of (a) preparing a candidate mixture of nucleic acids, (b) partitioning between members of said candidate mixture on the basis of affinity to the *Taq* or *Tth* polymerases and (c) amplifying the selected molecules using the target molecule to yield a mixture of nucleic acids enriched for nucleic acid sequences with a relatively higher affinity for binding to the *Taq* and *Tth* polymerases only at temperatures below the temperature of amplification, respectively.

The present invention, therefore, includes methods for identification of nucleic acid switches. Nucleic acids switches are nucleic acids identified by the SELEX process wherein the desired property of the nucleic acid can be "switched" on or off depending on the manipulation of some environmental parameter. Nucleic acid switches may be identified by manipulating the SELEX partitioning step to select for nucleic acids that give opposite results --

often binding to the target -- based on an alteration in a reaction medium parameter. The examples in this case demonstrate nucleic acid switches that are turned on and off based on temperature, however, the method of this invention can be extended to identifying and preparing nucleic ligands that
5 function as switches on the basis of conditions other than temperature, including but not limited to, pH, concentration of specific ions, ie. Mg^{++} .

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A shows the binding affinities of enriched pools of DNA after
10 12 rounds of SELEX (◦) and the unselected random pool (•) of DNA for the *Taq* polymerase. Figure 1B shows the binding affinities of enriched pools of DNA after 10 rounds of SELEX (◦) and the unselected random pool (•) of DNA for the *Tth* polymerase.

15 Figure 2A shows a cross binding analysis of the enriched DNA pool for the *Taq* polymerase (◦) and the enriched DNA pool for the *Tth* polymerase (•) to the *Tth* polymerase. Figure 2B shows a cross binding analysis of the enriched DNA pool for the *Taq* polymerase (◦) and the enriched DNA pool for the *Tth* polymerase (•) to the *Taq* polymerase.

20 Figure 3A depicts a binding curve for ligand 30 (•) and ligand 21 (◦) to *Taq* polymerase. Figure 3B depicts a binding curve for ligand 30 (•) and ligand 21 (◦) to *Tth* polymerase.

25 Figure 4 illustrates DNA polymerization.

Figure 5A illustrates a polymerase activity assay for the *Taq* and *Tth* polymerases carried out at different temperatures with different times of incubations. The DNA is resolved on a 15% polyacrylamide gel under

Figures 5D and 5E illustrate a third polymerase activity assay for the *Taq* and *Tth* polymerases, resolved on a 15% polyacrylamide gel under denaturing conditions. Figure 5D shows the activity of *Taq* polymerase in the presence of the enriched pool that has not been subjected to thermal cycling, whereas Figure 5E exhibits the activity of *Taq* polymerase in the presence of the enriched pool that has been thermal cycled. Lanes 1-5 indicate the amount of product formed over 5 minute incubations at 20°C, 25°C, 30°C, 35°C and 40°C, respectively. Lanes 6-10 exhibit *Taq* polymerase activity in the presence of the enriched pool over 5 minute incubations at 20°C, 25°C, 30°C, 35°C and 40°C, respectively. The schematics on right depict the starting short end-labeled DNA and the polymerase extended product.

Figure 6 depicts the effect of temperature on the inhibition of *Taq* polymerase (Figure 6A) and *Tth* polymerase (Figure 6B) by ligands TQ30 (SEQ ID NO:50) and TQ21 (SEQ ID NO:59) (lanes 1-10). The DNA is resolved on a 10% polyacrylamide gel under denaturing conditions. Lanes 11-15 depict the formation of product in the absence of an inhibitor. The right side of the autoradiograms schematically depict the 5'-labeled template before and after polymerase extension. Figures 6C and 6D show the percent of product formed in the presence of ligand TQ21 (○) and ligand TQ30 (●) using *Taq* polymerase (Figure 6C) and *Tth* polymerase (Figure 6D), respectively. The amount of product was quantitated by phosphorimager and normalized to the product formed in the absence of an inhibitor at the same temperature to obtain the percent of product (Figures 6C and 6D (abscissa)).

25

Figure 7 illustrates the reversible inhibition of *Taq* polymerase by ligand TQ30 (SEQ ID NO:50). The DNA is resolved on a 10% polyacrylamide gel under denaturing conditions. Lanes 1-5 show the products obtained in the absence of any inhibitor upon incubation between 20°C-40°C.

denaturing conditions. The data on Panel A were obtained with the *Taq* polymerase and the enriched pool selected for *Taq* polymerase, whereas those shown on Panel B were obtained with the *Tth* polymerase and the enriched pool selected for *Tth* polymerase. The untreated, 5'-end labeled DNA hairpin template (lane 1); the labeled template in a reaction mixture that lacks the polymerase (lane 2); incubation of the complete reaction mixture for 25 minutes at room temperature in the absence of (lane 3) and in the presence of the enriched pool (lane 4). Lanes 5, 6, and 7 show the incubations of complete reaction mixtures in the presence of the enriched pool for 5 minutes at 37°C, 50°C and 60°C, respectively. Lanes 8 and 9 show the incubations of the complete reaction mixtures in the presence (lane 8) and absence (lane 9) of the enriched pool at 70°C for 5 minutes. Lane 10 shows the gel mobility of the end-labeled pool DNA. The schematics on the right of the gels depict the positions of the starting short end-labeled DNA and the polymerase extended product.

Figures 5B and 5C illustrate a second polymerase activity assay for the *Taq* and *Tth* polymerases, performed at three different temperatures. The DNA is resolved on a 15% polyacrylamide gel under denaturing conditions. The data in Figure 5B were obtained with the *Taq* polymerase and the data in Figure 5C were obtained with the *Tth* polymerase. Lanes 1-3 show the products obtained in the absence of any inhibitor upon incubation at room temperature, 30°C and 37°C, respectively, for 5 minutes. Lanes 4-6 show the data obtained with the unselected random sequence pool; lanes 7-9 with the enriched pool for *Taq* polymerase; lanes 10-12 with the enriched pool for *Tth* polymerase; lanes 13-15 with *Taq*start antibody for 5 minute incubations at the three temperatures indicated. The schematics on the right indicate the starting short end-labeled DNA and the polymerase extended product.

Lanes 6-10 show the products formed upon incubation between 20°C-40°C in the presence of ligand TQ30 that had not been thermocycled (Figure 7A) and ligand TQ30 that had been subjected to 25 rounds of thermocycling.

5 Figure 8 depicts the effect of ligand concentration on the inhibition of *Taq* polymerase (Figure 8A) and *Tth* polymerase (Figure 8B) by ligands TQ30 (SEQ ID NO:50) (•) and TQ21 (SEQ ID NO:59) (◊). The amount of product formed in the presence of varying concentrations of inhibitor in the template extension assays was quantitated by phosphorimager and normalized to the
10 amount of product formed in the absence of an inhibitor to obtain the percent product (abscissa).

 Figure 9 illustrates schematically cleavage of the 97-nucleotide DNA sequence (Exo-Sub)
15 (5'-TTCGAGCGTGAATCTGAATTCGCGGCTAGCCAGCTTTTGCTG
GCTAGCCGCGGTGGGAACTGAGGTAGGTGTTTTCACCTACCTCAG
TTTCCCACC-3' (SEQ ID NO:75)), with predicted two stem-loops with a
displaced strand, catalyzed by the 5'-3' exonuclease activity of *Taq* and *Tth*
polymerase. Polarity of the folded sequence is indicated by the small arrow.
20 The cleavage mediated by the exonuclease activity of the DNA polymerases is
expected to occur near the junction of the displaced strand and the helix,
resulting in two DNA fragments of 20-nucleotides and 77-nucleotides. Solid
circles at the two ends of the molecule indicate radiolabels.

25 Figure 10 illustrates the detection of a low copy number target using
standard PCR amplification, "hot start" PCR and PCR amplification in the
presence of oligonucleotide inhibitors TQ30 and TQ21 ("NeXstart PCR").
Figure 10A illustrates a comparison of amplification performed under standard
conditions (lanes 1-3) with those of "hot start" PCR (lanes 4-6) in detecting the

target at ~ 10 and 50 copies. Figure 10B illustrates a comparison PCR amplifications conducted in the presence of a nonspecific (NS) oligonucleotide (lanes 1-3) with those of TQ21 (lanes 4-6) and TQ30 (lanes 7-9) in detecting the target at ~10 and 50 copies. Figure 10C illustrates the detection of very low number target copies (as indicated) in the presence of oligonucleotide inhibitors TQ21 and TQ30. In both (B) and (C) oligonucleotide inhibitors were used at a concentration of 50 nM. M indicates molecular weight standards. Arrows in each panel shows the position of the target-specific 203-bp DNA in the gels.

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Figure 11 depicts the effect of the concentration of truncated ligands Trunc.1-30 (SEQ ID NO:75) (•), Trnc.2-30 (SEQ ID NO:76) (■) and Trnc.3-30 (SEQ ID NO:77) (▲) on the activity of *Taq* polymerase. The amount of product formed in the presence of varying concentrations of inhibitor was quantitated by phosphorimager and normalized to the amount of product formed in the absence of an inhibitor to obtain the percent product (abscissa).

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Figure 12 depicts the effect of inhibitor concentration of truncated ligands Trunc.1-30 (•), Trnc.2-30 (■) and Trnc.3-30 (▲) on the activity of the Stoffel fragment. The amount of product formed in the presence of varying concentrations of inhibitor was quantitated by phosphorimager and normalized to the amount of product formed in the absence of an inhibitor to obtain the percent product (abscissa).

20

Figure 13 illustrates the affinity and inhibition characteristics of truncated ligand Trnc.21 (SEQ ID NO:70). Figure 13A depicts a binding curve for ligand Trnc.21 to *Taq* polymerase. Figure 13B illustrates the effect of Trnc.21 concentration on the activity of *Taq* polymerase (•) and *Tth* polymerase (◊). IC_{50} values for *Taq* polymerase and *Tth* polymerase are 21

25

and 36.5 nM, respectively. Figure 13C depicts the effect of temperature on the inhibition of *Taq* polymerase (•) and *Tth* polymerase (◊) by Trnc.21. The amount of product formed in the presence of the inhibitor at a given temperature was normalized to that formed in the absence of an inhibitor at the same temperature to obtain the percent product. The calculated IT_{50} values for *Taq* polymerase and *Tth* polymerase are 34 °C and 35.6 °C, respectively.

Figure 14 depicts the affinity and inhibition characteristics of the homodimer (D.30-D.30) (SEQ ID NO:71). Figure 14A depicts a binding curve for homodimer (D.30-D.30) to *Taq* polymerase ($K_d = 47.5 \pm 5$ pM). Figure 14B illustrates the effect of dimeric (•) and monomeric (◊) ligand concentrations on the activity of *Taq* polymerase. The IC_{50} value of Trnc.2-30 (monomer) is 48 nM, whereas that of D.30-D.30 (dimer) is 14 nM.

Figure 15 depicts the inhibition characteristics of the heterodimer D.21-D.30 (SEQ ID NO:72). Figure 15A illustrates the effect of D.21-D.30 concentration on the activity of *Taq* polymerase (•) and *Tth* polymerase (◊). IC_{50} values for the inhibition of these two polymerases are approximately 30 nM. Figure 15B illustrates the effect of temperature on the inhibition of *Taq* polymerase (•) and *Tth* polymerase (◊) by heterodimer D.21-D.30. The IT_{50} value for *Taq* polymerase is 41°C, whereas that for *Tth* polymerase is 34.5°C.

Figure 16 illustrates the effect of dNTPs and the hairpin template DNA on the binding affinity of Trnc.21 to *Taq* polymerase. Figure 16A Nitrocellulose filter binding analysis of Trnc.21 in the presence of 1 mM dNTPs. Closed circles (•) indicate the binding in the absence of hairpin DNA template, whereas open circles (◊) indicate the binding in the presence of 250 nM hairpin DNA template. The calculated K_d values under these conditions are approximately 2.5 nM. Figure 16B illustrates the effect of dNTP

concentration on the binding of Trnc.21 to *Taq* polymerase. In this experiment binding of the radiolabeled Trnc.21 to 1 nM *Taq* polymerase was monitored in the presence of varying concentration of dNTPs.

5 **DETAILED DESCRIPTION OF THE INVENTION**

This application describes the isolation of nucleic acid ligands to DNA polymerases. Specifically, this application describes the isolation of nucleic acid ligands to thermostable polymerases useful in the Polymerase Chain Reaction. In a preferred embodiment the DNA polymerase is selected from
10 *Taq* or *Tth* polymerase, however the method of this invention can be extended to the identification and purification of high-affinity nucleic acid ligands to any thermostable DNA polymerase. The nucleic acid ligands are identified through the method known as SELEX. SELEX is described in United States Patent Application Serial No. 07/536,428, entitled Systematic Evolution of
15 Ligands by EXponential Enrichment, now abandoned, United States Patent Application Serial No. 07/714,131, filed June 10, 1991, entitled Nucleic Acid Ligands, now United States Patent No. 5,475,096 and United States Patent Application Serial No. 07/931,473, filed August 17, 1992, entitled Nucleic Acid Ligands, now United States Patent No. 5,270,163, (see also
20 PCT/US91/04078). These applications, each specifically incorporated herein by reference, are collectively called the SELEX Patent Applications.

In its most basic form, the SELEX process may be defined by the following series of steps:

- 1) A candidate mixture of nucleic acids of differing sequence is
25 prepared. The candidate mixture generally includes regions of fixed sequences (*i.e.*, each of the members of the candidate mixture contains the same sequences in the same location) and regions of randomized sequences. The fixed sequence regions are selected either: (a) to assist in the amplification steps described below, (b) to mimic a sequence known to bind to the target, or

(c) to enhance the concentration of a given structural arrangement of the nucleic acids in the candidate mixture. The randomized sequences can be totally randomized (*i.e.*, the probability of finding a base at any position being one in four) or only partially randomized (*e.g.*, the probability of finding a base at any location can be selected at any level between 0 and 100 percent).

2) The candidate mixture is contacted with the selected target under conditions favorable for binding between the target and members of the candidate mixture. Under these circumstances, the interaction between the target and the nucleic acids of the candidate mixture can be considered as forming nucleic acid-target pairs between the target and those nucleic acids having the strongest affinity for the target.

3) The nucleic acids with the highest affinity for the target are partitioned from those nucleic acids with lesser affinity to the target. Because only an extremely small number of sequences (and possibly only one molecule of nucleic acid) corresponding to the highest affinity nucleic acids exist in the candidate mixture, it is generally desirable to set the partitioning criteria so that a significant amount of the nucleic acids in the candidate mixture (approximately 5-50%) are retained during partitioning.

4) Those nucleic acids selected during partitioning as having the relatively higher affinity to the target are then amplified to create a new candidate mixture that is enriched in nucleic acids having a relatively higher affinity for the target.

5) By repeating the partitioning and amplifying steps above, the newly formed candidate mixture contains fewer and fewer unique sequences, and the average degree of affinity of the nucleic acids to the target will generally increase. Taken to its extreme, the SELEX process will yield a candidate mixture containing one or a small number of unique nucleic acids representing those nucleic acids from the original candidate mixture having the highest affinity to the target molecule.

The SELEX Patent Applications describe and elaborate on this process in great detail. Included are targets that can be used in the process; methods for partitioning nucleic acids within a candidate mixture; and methods for amplifying partitioned nucleic acids to generate enriched candidate mixture.

- 5 The SELEX Patent Applications also describe ligands obtained to a number of target species, including both protein targets where the protein is and is not a nucleic acid binding protein.

The SELEX process provides high affinity ligands of a target molecule. This represents a singular achievement that is unprecedented in the field of
10 nucleic acids research. The present invention applies the SELEX procedure to the specific targets of nucleic acid inhibitors of DNA polymerases, particularly the *Taq* and *Tth* polymerases. In the Example section below, the experimental parameters used to isolate and identify the nucleic acid inhibitors to the *Taq* and *Tth* polymerases are described.

- 15 In co-pending and commonly assigned United States Patent Application Serial No. 07/964,624, filed October 21, 1992 ('624), now United States Patent No. 5,496,938, methods are described for obtaining improved nucleic acid ligands after SELEX has been performed. The '624 application, entitled Methods of Producing Nucleic Acid Ligands, is specifically incorporated
20 herein by reference.

Certain terms used to describe the invention herein are defined as follows:

- "Nucleic Acid Ligand" as used herein is a non-naturally occurring nucleic acid having a desirable action on a target. A desirable action includes,
25 but is not limited to, binding of the target, catalytically changing the target, reacting with the target in a way which modifies/alters the target or the functional activity of the target, covalently attaching to the target as in a suicide inhibitor, facilitating the reaction between the target and another molecule. In the preferred embodiment, the action has specific binding affinity

for a target molecule, such target molecule being a three dimensional chemical structure other than a polynucleotide that binds to the nucleic acid ligand through a mechanism which predominantly depends on Watson/Crick base pairing or triple helix binding, wherein the nucleic acid ligand is not a nucleic acid having the known physiological function of being bound by the target molecule. Nucleic acid ligands include nucleic acids that are identified from a candidate mixture of nucleic acids, said nucleic acid ligand being a ligand of a given target by the method comprising: a) contacting the candidate mixture with the target, wherein nucleic acids having an increased affinity to the target relative to the candidate mixture may be partitioned from the remainder of the candidate mixture; b) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture; and c) amplifying the increased affinity nucleic acids to yield a ligand-enriched mixture of nucleic acids.

"Candidate Mixture" is a mixture of nucleic acids of differing sequence from which to select a desired ligand. The source of a candidate mixture can be from naturally-occurring nucleic acids or fragments thereof, chemically synthesized nucleic acids, enzymatically synthesized nucleic acids or nucleic acids made by a combination of the foregoing techniques. In a preferred embodiment, each nucleic acid has fixed sequences surrounding a randomized region to facilitate the amplification process.

"Nucleic Acid" means either DNA, RNA, single-stranded or double-stranded and any chemical modifications thereof. Modifications include, but are not limited to, those which provide other chemical groups that incorporate additional charge, polarizability, hydrogen bonding, electrostatic interaction, and fluxionality to the nucleic acid ligand bases or to the nucleic acid ligand as a whole. Such modifications include, but are not limited to, 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at exocyclic amines, substitution of 4-thiouridine, substitution of 5-bromo or 5-iodo-uracil,

backbone modifications, methylations, unusual base-pairing combinations such as the isobases isocytidine and isoguanidine and the like. Modifications can also include 3' and 5' modifications such as capping.

"SELEX" methodology involves the combination of selection of
5 nucleic acid ligands which interact with a target in a desirable manner, for example binding to a protein, with amplification of those selected nucleic acids. Iterative cycling of the selection/amplification steps allows selection of one or a small number of nucleic acids which interact most strongly with the target from a pool which contains a very large number of nucleic acids.
10 Cycling of the selection/amplification procedure is continued until a selected goal is achieved. In the present invention, the SELEX methodology is employed to obtain nucleic acid ligands to the *Taq* and *Tth* polymerases.

The SELEX methodology is described in the SELEX Patent Applications.

15 "Target" means any compound or molecule of interest for which a ligand is desired. A target can be a protein, peptide, carbohydrate, polysaccharide, glycoprotein, hormone, receptor, antigen, antibody, virus, substrate, metabolite, transition state analog, cofactor, inhibitor, drug, dye, nutrient, growth factor, etc. without limitation. In this application, the target is
20 a DNA polymerase. In a preferred embodiment the DNA polymerase is *Taq* polymerase and *Tth* polymerase.

A "Labile ligand" as used herein is a nucleic acid ligand identified by the SELEX process that has a greatly decreased affinity for its target based on an adjustment of an environmental parameter. In the preferred embodiment,
25 the environmental parameter is temperature, and the affinity of a ligand to its target is decreased at elevated temperatures.

"DNA Polymerase" as used herein refers to any enzyme which catalyzes DNA synthesis by addition of deoxyribonucleotide units to a DNA chain using DNA or RNA (reverse transcriptase) as a template. Thermostable

DNA polymerases are isolated from microorganisms which thrive in temperatures greater than 40°C.

A "Switch" refers to any compound which functions to turn a reaction "on" or "off" depending upon some specific reaction condition(s). In the present invention the nucleic acid ligands function to turn the PCR "on" or "off" depending upon the temperature of the reaction. A switch can operate on the basis of other reaction conditions including pH, ionic strength or the presence or absence of specific ions. Nucleic acid switches are identified via the SELEX method by the appropriate selection of partitioning techniques. Partitioning parameters are determined in order that nucleic acids are selected that have the desired switching characteristics.

In the present invention, a SELEX experiment was performed in order to identify nucleic acid ligands with specific high affinity for the *Taq* and *Tth* polymerases from a degenerate library containing 30 random positions (30N) (Example 1). Although RNA or DNA ligands could be identified for this purpose, the examples below describe the identification of DNA ligands. The SELEX experiment was designed to identify oligonucleotides that bind and inhibit the polymerases at low temperature (room temperature), but not at higher temperatures (>40°C). This was accomplished by using the target polymerase to amplify affinity-selected molecules in PCR at an elevated temperature. Under such conditions, DNA sequences that inhibit the *Taq* and *Tth* polymerases at high temperature were not expected to amplify and propagate during selection. This invention includes the specific ssDNA ligands to *Tth* polymerase shown in Table 2 (SEQ ID NOS:7-35) and *Taq* polymerase shown in Table 3 (SEQ ID NOS:36-66, 76, 77) and the nucleic acid ligands shown in Tables 4 and 5 (SEQ ID NO:67-74), identified by the methods described in Example 1. This invention further includes DNA ligands to *Taq* and *Tth* polymerase that inhibit the function of *Taq* and *Tth* polymerase.

The scope of the ligands covered by this invention extends to all nucleic

acid ligands of the *Taq* and *Tth* polymerases, modified and unmodified, identified according to the SELEX procedure. More specifically, this invention includes nucleic acid sequences that are substantially homologous to the ligands shown in Tables 2-5. By substantially homologous it is meant a degree of primary sequence homology in excess of 70%, most preferably in excess of 80%. A review of the sequence homologies of the ligands of *Taq* and *Tth* shown in Tables 2-5 shows that sequences with little or no primary homology may have substantially the same ability to bind *Taq* and *Tth* polymerase, respectively. For these reasons, this invention also includes nucleic acid ligands that have substantially the same ability to bind the *Taq* and *Tth* polymerases as the nucleic acid ligands shown in Tables 2-5. Substantially the same ability to bind *Taq* or *Tth* polymerase means that the affinity is within a few orders of magnitude of the affinity of the ligands described herein. It is well within the skill of those of ordinary skill in the art to determine whether a given sequence -- substantially homologous to those specifically described herein -- has substantially the same ability to bind *Taq* and *Tth* polymerase, respectively.

This invention also includes the ligands as described above, wherein said ligands inhibit the function of other thermostable DNA polymerases, including, but not limited to, the Stoffel fragment, *Tbr* polymerase, *Tlf* polymerase and M-MLV reverse transcriptase.

This invention also includes the ligands as described above, wherein certain chemical modifications are made in order to increase the *in vivo* or *in vitro* stability of the ligand or to enhance or mediate the binding or other desirable characteristics of the ligand or the delivery of the ligand. Examples of such modifications include chemical substitutions at the sugar and/or phosphate and/or base positions of a given nucleic acid sequence. See, e.g., United States Patent Application Serial No. 08/117,991, filed September 9, 1993, entitled High Affinity Nucleic Acid Ligands Containing Modified

Nucleotides which is specifically incorporated herein by reference. Other modifications are known to one of ordinary skill in the art. Such modifications may be made post-SELEX (modification of previously identified unmodified ligands) or by incorporation into the SELEX process.

5 The nucleic acid ligands to the *Taq* and *Tth* polymerases described herein are useful as reagents in the Polymerase Chain Reaction.

 The present invention includes an improved method for performing the Polymerase Chain Reaction, wherein a sample containing a nucleic acid sequence that is to be amplified is mixed with 1) primers that are
10 complementary to sequences that flank the sequence to be amplified, 2) a thermostable polymerase, and 3) a nucleic acid ligand that is capable of inhibiting the polymerase at ambient temperatures. The nucleic acid ligand inhibitor may be immobilized on a solid support. The normal steps of PCR are then followed -- melting, annealing and synthesis -- by thermal cycling of the
15 mixture. The presence of the nucleic acid ligand prevents the mixture from amplifying background DNA by preventing any synthesis at lowered temperatures prior to or during cycling. The present invention also includes a PCR kit comprising a thermostable DNA polymerase and a nucleic acid ligand that inhibits said polymerase at ambient temperatures, yet allows synthesis to
20 occur during the elevated temperature cycles of the PCR process. The present invention also includes a method for improving PCR, as understood by those skilled in the art, including the step of adding to the thermostable polymerase a nucleic acid ligand that inhibits said polymerase at ambient temperatures yet allows synthesis to occur during the elevated temperature cycles of the PCR
25 process.

Nucleic Acid Ligands to *Taq* and *Tth* Polymerase.

Example 1 describes the experimental procedures used in the selection of nucleic acid ligands to both the *Taq* and *Tth* polymerases. The ss-DNA sequences obtained from 10 rounds of selection performed with *Tth* polymerase

are set forth in Table 2. Twenty nine individual clones were sequenced from the *Tth* polymerase selection (only the variable 30 nucleotide region is shown in Table 2). The ligands were grouped into families based upon primary sequence homology.

- 5 The ss-DNA sequences obtained from 12 rounds of selection performed with *Taq* polymerase are set forth in Table 3. Of forty two sequences analyzed from the *Taq* polymerase selection, thirty three were unique. The upper case letters depict the 30-nucleotide random region that is flanked by the 5'-TTCTCGGTTGGTCTCTGGCGGAGC- and -TCTTGTGTATGATTC
- 10 GCTTTTCCC-3' fixed sequence regions to form full-length sequences. The lowercase letters in some of the sequences depict the 5'-fixed sequence. The number of clones carrying the same sequence is indicated in parenthesis. The sequences were grouped into three families based on sequence similarity. Conserved sequence motifs in families I and II are boxed. Both families
- 15 contained a different consensus sequence; 5'-A/GA₆TGT G_{1A}CAGTAT/GC-3' for Family I and 5'-A₆CGTTTTTG-3' for Family II. In Family I, the 5' and the 3' regions of the consensus sequence showed potential for base pairing with each other (underlined in Table 3). Additionally, the covariation observed in these regions suggests the existence of a possible stem loop structure. In most
- 20 of the ligands the potential base pairing regions extend beyond the consensus region. In contrast, Family II ligands do not have an obvious secondary structural motif.

- Representative binding curves of clone 30 (TQ30 (SEQ ID NO:50)) from Family I and clone 21 (TQ21 (SEQ ID NO:59)) from Family II, are
- 25 shown in Figure 3. In both cases, the ligands show tight binding to the two polymerases, with K_d values in the low picomolar range; K_d values of TQ30 are 40 ± 1 pM for *Taq* polymerase and 28 ± 4 pM for *Tth* polymerase, whereas those of TQ21 are 36 ± 4 pM and 10 ± 2 pM for *Taq* polymerase and *Tth* polymerase, respectively. Several more ligands from the two families were

screened. K_d values ranged from 0.04 to 9 nM for *Taq* polymerase and from 0.01 to 0.3 nM for *Tth* polymerase.

Polymerase Inhibition Assays: *Taq* and *Tth* Polymerase.

Example 2 (Figures 5-9) describes a number of polymerase inhibition
5 assays and demonstrates that the ligands of the invention are capable of
inhibiting the interaction of both the *Taq* and *Tth* polymerases, at temperatures
less than 40°C. In Example 2, the designed hairpin DNA (DNA-HP;
5'-ATGCCTAAGTTTCGAACGCGGCTAGCCAGCTTTT
GCTGGCTAGCCGCGT-3' (SEQ ID NO:6) is used as a template for
10 measurement of the ability of the enriched pools of DNA, as well as, ligands
TQ30 (SEQ ID NO:50) and TQ21 (SEQ ID NO:59) from the *Taq* polymerase
selection, to inhibit polymerase activity, under a variety of conditions. This
assay detects template-directed fill-in synthesis of 15 nucleotides on a
fold-back DNA hairpin.

15 Figure 5A shows the results of inhibition assays carried out at different
temperatures with different times of incubations using the enriched pools of
DNA ligands. The activity of both the *Taq* and *Tth* polymerases is generally
low at low temperatures and increases as the temperature is increased, as can
be seen by comparing lane 3 (room temperature reaction) with lanes 6-9
20 (reaction at 50, 60 and 70°C, respectively). The enriched pools inhibit the
activity of their respective polymerases at room temperature (lane 4), but not at
50°C-70°C. Lane 10 shows the mobility of the radiolabeled pool as a
reference to detect the possible extension of DNA molecules in the pool that
can serve as a template for the polymerases. The lack of radiolabeled bands
25 migrating closer or above the labeled pool in lanes 6-9 indicates the absence of
polymerization of the ssDNA pool.

Since the activity of thermostable polymerases is low at ambient
temperature, the incubation period in the assay was increased to 16 hours.

Figures 5B and 5C show the results of 16 hour incubation of the template with

the two polymerases in the presence of selected pools and the random pool. In addition, the inhibition mediated by selected pools was compared to that of anti-*Taq* antibody (*TaqStart*). The data in Figure 5B was obtained with the *Taq* polymerase and the data in Figure 5C was obtained with the *Tth* polymerase. Over the three temperatures studied, room temperature, 30°C and 37°C, the random pool did not show inhibition of the two polymerases (compare lanes 1-3 with 4-6), suggesting that the inhibition caused by the enriched pool is sequence specific. The pool selected for *Taq* polymerase completely inhibited the polymerase activity over a 16 hour incubation only at room temperature (lane 7), but not at 30°C and above (lanes 8 & 9). Although the pool selected for *Tth* polymerase did show binding to *Taq* polymerase, it was unable to inhibit *Taq* polymerase (lanes 10-12). As expected, *Taqstart* antibody inhibited the polymerase activity at all three temperatures investigated (lanes 12-15). The ssDNA pool selected for *Tth* polymerase, however, did not inhibit the enzyme activity over a 16 hour incubation (compare lanes 1-3 with 4-6). In contrast, the same pool was able to inhibit the enzyme activity over short periods of incubation. The pool selected for *Taq* polymerase was able to partially inhibit (> 50%) the *Tth* activity over 16 hour incubation at room temperature (lane 10). *Taqstart* antibody did not have any effect on the activity of *Tth* (lanes 13-15).

The use of *Taqstart* antibody is limited to one time in a PCR reaction. Once it is denatured at high temperature it cannot renature back to its native form. Nucleic acid ligands with simple secondary structures, however, have the potential to renature back to their native form after going through a thermal cycle. An experiment was carried out to investigate whether the inhibitory capacity of the DNA pool selected for *Taq* polymerase can be restored after heating (Figures 5D and 5E). Figure 5D shows the inhibition of *Taq* activity between 20°C- 40°C by the selected DNA pool that has not been subjected to thermocycling. Over 45 minutes of incubation, the pool completely inhibits

Taq activity at 20°C and 25°C. Within this relatively short period of incubation, the pool exhibited >70% inhibition at 30°C. A very similar inhibition profile can be seen with the DNA pool that has been subjected to two PCR cycles with the *Taq* polymerase in the absence of the template DNA.

- 5 This result demonstrates that the inhibition mediated by ssDNA is reversibly temperature sensitive and can be restored even after PCR.

Figure 6 shows the temperature range in which sequences, TQ30 (SEQ ID NO:50) and TQ21 (SEQ ID NO:59) (Table 3), are inhibitory toward the *Taq* and *Tth* DNA polymerases. The hairpin extension assays depicted in this figure were performed at the indicated temperatures for 1 hour using 250 nM of the respective ligand (lanes 1-10). As anticipated, the ssDNA ligands did not inhibit either DNA polymerase at temperatures >40°C (Figures 6A and 6B). The temperatures at which 50% of the product is generated during the one-hour assay (IT_{50} values) for ligand TQ30 are 41°C and 29°C for *Taq* polymerase and *Tth* polymerase, respectively. The respective values for ligand TQ21 are 37°C and 29°C. Binding affinities of the two ligands for these polymerases decrease at higher temperatures (data not shown), in agreement with their decreased inhibitory activity at high temperature. In the hairpin extension assays, approximately 2% of the input hairpin template was not extended by DNA polymerase, presumably due to incorrect folding.

Figure 7 illustrates that the inhibition of *Taq* polymerase by ligand TQ30 (SEQ ID NO:50) is thermally reversible and can be restored even after PCR. The hairpin template extension assays depicted in this figure were performed at the indicated temperatures for 10 minutes in a 100 µL reaction volume with 5 U of *Taq* polymerase, in the absence (lanes 1-5) and in the presence of ligand TQ30 (50 nM) (lanes 6-10). In Figure 7A, ligand TQ30 had not been subjected to thermocycling. In Figure 7B, ligand TQ30 was subjected to 25 rounds of thermocycling with *Taq* polymerase (30 seconds at 90°C; 1 minute at 50°C, 30 seconds at 72°C) and cooled to room temperature

before adding the radiolabeled hairpin template (250 nM). As can be seen in Figure 7, in both cases ligand TQ30 inhibited the polymerase at temperatures below 40°C. Additionally, the sample that underwent thermocycling showed identical or more effective inhibition than the sample not subjected to thermocycling.

Figure 8 demonstrates the effect of ligand concentration on the inhibition of the *Taq* and *Tth* polymerases. The concentration of inhibitor required to produce 50% of the product in the hairpin assay (IC_{50} values) for TQ30 (SEQ ID NO:50) and TQ21 (SEQ ID NO:59) were 6.5 nM and 10 nM, respectively, for inhibition of *Taq* polymerase at room temperature (approximately 22°C) over a 16 hour incubation (Figure 8A). Since the concentration of *Taq* polymerase used in the assay is 12.5 nM, enzyme inhibition by TQ30 (SEQ ID NO:50) is likely to be a result of stoichiometric binding. When assayed at 30°C over 1 hour, IC_{50} values increased by approximately three fold (22 nM for TQ30 and 67 nM for TQ21; data not shown). The IC_{50} values of TQ30 and TQ21 for the inhibition of *Tth* polymerase were 60 and 36 nM, respectively, at room temperature (Figure 8B). Overall, these oligonucleotides are more effective inhibitors for *Taq* polymerase, the enzyme used in selection, than for *Tth* polymerase.

To rule out the possibility that the observed inhibition of the extension of the template is due to preferential binding of selected ligands to the polymerase and subsequent utilization as substrates, 5'-end radiolabeled TQ21 and TQ30 ligands were incubated with the two DNA polymerases for 16 hours (Example 2, data not shown). Ligand TQ30 did not show extension products upon incubation with either enzyme, indicating that it is not a substrate for the polymerase activity. TQ21, however, gave a higher molecular weight band indicating sequence extension upon incubating with both polymerases. The observed partial extension of ligand TQ21 was effectively eliminated by blocking the availability of the 3' OH group by capping the 3' end with an

ethylene glycol linker using standard conditions. The 3'-capped oligonucleotide constructs are equally effective inhibitors as the uncapped molecules (data not shown). These results indicate that the ssDNA ligands are poor substrates for polymerase activity and that the two types of ligands are likely positioned on DNA polymerases differently; TQ21 binds to the polymerases such that its 3' end can be extended (albeit poorly), whereas TQ30 cannot extended upon binding.

Affinity Capture Experiment.

The thermal reversibility of the interaction of nucleic acid ligands with the *Taq* and *Tth* polymerases raises the possibility of the use of an affinity matrix generated with such ligands, to capture the polymerase after one amplification, for reuse in a subsequent amplification. To investigate the possibility of affinity capture, affinity beads containing ligands TQ30 (SEQ ID NO:50) and TQ21 (SEQ ID NO:59) were prepared as described in Example 1. After extension of the hairpin template with *Taq* and *Tth* polymerases in a PCR buffer containing heparin the reaction was mixed with either affinity beads or control beads as described in Example 2, the beads were washed thoroughly and then exposed to a fresh aliquot of reaction mixture containing all of the reagents, except the polymerase. After incubating for an additional 5 minutes at 70°C to allow the extension on the newly added template, the reaction mixtures were analyzed on an 8% polyacrylamide gel under denaturing conditions. In reaction mixtures that contained the control beads there is no extension of the template in the second round of amplification. In contrast, there is no difference in the extension products in both first and the second rounds of amplification in the reaction mixtures that contained affinity beads, indicating that the affinity beads containing both, ligand TQ30 (SEQ ID NO:50) and TQ21, successfully captured the two polymerases after the first round of PCR.

Effect of Ligands TQ30 and TQ21 on the Exonuclease Activity of *Taq* and *Tth* Polymerase.

As discussed above, in addition to their ability to catalyze polynucleotide synthesis, both *Taq* and *Tth* polymerase also possess 5'-3' exonuclease activity (Joyce and Steitz (1987) Trends Biochem. Sci. 12:288; Longley *et al.* (1990) Nucleic Acids Res. 18:7317). The preferred substrate for the 5'-3' exonuclease activity is a displaced ssDNA (or a fork-like structure) with cleavage occurring near the duplex/ssDNA junction. To study the effect of the oligonucleotide inhibitors on the 5'-3' exonuclease activity of the polymerases, DNA substrate (Exo-Sub) containing a displaced ssDNA in a hairpin was designed (Example 3, Figure 9). Radiolabeling the Exo-Sub substrate at both the 5' and 3' ends allowed detection of the two DNA fragments produced by the exonuclease activity. The two labeled DNA fragments originating from the exonuclease activity appeared both in the presence and absence of the oligonucleotide inhibitors (data not shown), however, the amount of cleavage products generated in the presence of the oligonucleotide inhibitors was somewhat lower than that produced in the absence of inhibitors, indicating that oligonucleotide inhibitors exert some inhibitory effect toward the exonuclease activity of the enzymes. Since these oligonucleotides completely inhibited the polymerase activities of the two enzymes at 250 nM, their effect on exonuclease activity is considered marginal.

Inhibition of other DNA Polymerases.

Inhibition assays using several other commercially available DNA polymerases and ligands TQ21 (SEQ ID NO:59) and TQ30 (SEQ ID NO:50) as inhibitors are described in Example 4. Four thermostable enzymes (*Tbr* polymerase from *Thermus brockianus*, *Tfl* polymerase from *Thermus flavus*, *Tma* polymerase from *Thermotoga maritima* and *Tli* polymerase from *Thermococcus litoralis*); three mesophilic enzymes (Klenow fragment of

E. coli DNAP1 (KF), T4 DNA polymerase and T7 DNA polymerase); and four reverse transcriptases (RT) (HIV-I RT, AMV (avian myeloblastosis virus) RT and M-MLV (moloney murine leukemia virus) RT and its mutant lacking RNase H activity (SuperScript II) were examined.

5 Of the six thermostable polymerases examined (including *Taq* and *Tth* polymerase), the four polymerases derived from *Thermus* species (*Taq*, *Tth*, *Tbr* and *Tfl*) were inhibited by both of the selected oligonucleotides, suggesting that these enzymes share a high degree of similarity. As stated above, *Tth* polymerase and *Taq* polymerase are reported to be 93% similar and
10 88% identical at the amino acid sequence level (Abramson (1995) in PCR Strategies (Academic Press, New York). *Tfl* polymerase is reported to be 93% similar and 86% identical to *Taq* polymerase at the amino acid level (D. Gelfand, personal communication). *Tma* polymerase from *Thermotoga maritima* and *Tli* polymerase from *Thermococcus litoralis*, on the other hand,
15 were not inhibited by either of the ligands. *Tli* polymerase shares little sequence homology with eubacterial enzymes (Ito and Braithwaite (1991) *Nucleic Acids Res.* 19:4045). *Tma* polymerase is reported to be 61% similar and 44% identical to *Taq* polymerase at the amino acid level (Abramson (1995) in PCR Strategies (Academic Press, New York), yet the oligonucleotide
20 ligands do not inhibit *Tma* polymerase.

 Of the four reverse transcriptases tested, RTs from HIV-I and AMV (avian myeloblastosis virus) were not inhibited. On the other hand, RT from M-MLV (moloney murine leukemia virus) and its mutant lacking RNase H activity (SuperScript II) were inhibited by the two oligonucleotide ligands.

25 Mesophilic DNA polymerases, such as, Klenow fragment of *E. coli* DNAP1 (KF), T4 DNAP and T7 DNAP were not inhibited by either ligand at 0.5 μ M concentration, despite the similarity of the polymerase domains of *Taq* polymerase and KF (Kim *et al.* (1995) *Nature* (London) 376:612; Lawyer *et al.* (1989) *J. Biol. Chem.* 264: 6427). Thus, it appears that the oligonucleotide

inhibitors are generally fairly specific. These results are similar to the behavior of nucleic acid ligands identified by *in vitro* selection for other Reverse transcriptases (Tuerk and MacDougall (1994) Proc. Natl. Acad. Sci, U.S.A. **89**:6988; Chen and Gold (1994) Biochemistry **33**:8746; Schneider *et al.* (1995) Biochemistry **34**:9599).

Amplification of Low Copy Number Targets.

Example 5 (Figure 10) describes a number of PCR amplifications comparing standard PCR techniques, "hot start" PCR and PCR using the TQ30 and TQ21 to facilitate the detection of a low copy number target by PCR in the absence of "hot start" conditions. A primer-template system designed to detect a 203-base pair (bp) DNA fragment from the HIV-2 LTR (long terminal repeat) as described by Respass *et al.* (1994) in Interscience Conference on Antimicrobial Agents and Chemotherapy **94**:110 was utilized. The PCR amplifications were carried out with 0, 10 and 50 copies of HIV-2 LTR target. Under normal PCR conditions, the identification of the correct target band was compromised by the presence of a number of nonspecific bands (Figure 10A, lanes 1-3). Amplification carried out under "hot start" conditions eliminated the nonspecific bands (Figure 10A, lanes 4-6). The results of amplification performed in the presence of a nonspecific 78-nucleotide ssDNA sequence containing identical 5'- and 3'-fixed sequences as TQ21 and TQ30 (Figure 10B, lanes 1-3) were similar to those obtained by PCR without using "hot start" conditions. However, the addition of either TQ21 (Figure 10B, lanes 4-6) or TQ30 (Figure 10B, lanes 7-9) carried out under standard conditions (without "hot start") eliminated the nonspecific bands without affecting the yield of the target-specific band. Of particular importance was the observation that when the target copy number was low, signal detection was very efficient (Figure 10B, compare lane 2 with lanes 5 and 8). The effect of oligonucleotide inhibitors was similar when *Tth* polymerase was used in place of *Taq* polymerase (data not shown) in detecting low copy number HIV-2 LTR. The

enhanced yield of the target-specific band obtained with the oligonucleotide inhibitors in PCR increases the sensitivity of the reaction, facilitating detection of the target present with only approximately 3 copies (Figure 10C).

The oligonucleotide inhibitors used in the experiment described in Figure 10 were uncapped at their 3' ends, potentially permitting them to initiate amplification nonspecifically, and further complicating the outcome of PCR. However, no adventitious bands were detected, suggesting that in this system, 3'-capping of oligonucleotide inhibitors was not required to eliminate the generation of nonspecific bands.

10 Identification of Truncated Ligands of TQ30 and TQ21 with Inhibitory Activity.

Typically, not all nucleotides in a full-length sequence are necessary for its function. Identification of truncated DNA sequences that retain the function of the whole sequence, therefore, is desirable. Ligands TQ30 (SEQ ID NO:50) from Family I and TQ21 (SEQ ID NO:59) from Family II (see Table 4), were chosen for truncation experiments. Affinity selections on end-labeled nested fragments generated from the full-length sequences of both ligands, followed by sequencing gel analysis, as described in Example 2, did not give identifiable boundaries. The two ligands were therefore subjected to deletion analysis. Sequentially deleted forms were tested for their ability to inhibit polymerases in the hairpin extension assay to identify functional truncates.

Truncates of ligand TQ30 (SEQ ID NO:50).

The variable 30-nucleotide region of TQ30 containing the conserved sequence motif with the predicted stem-loop structure (Trnc.A-30 (SEQ ID NO:74); Table 5) inhibits *Taq* polymerase at 25°C to the same extent as the full-length sequence (data not shown). At higher temperatures, however, the efficiency of inhibition is lower than the full-length sequence. At 30°C, for example, the inhibition of *Taq* polymerase by Trnc.A-30 (250 nM) is

approximately 82%, whereas the full-length sequence completely inhibited the enzyme at this temperature and concentration. The increased thermal sensitivity of Trnc.A-30 may be due to the presence of an interrupted helix with A-T base pairs, a helix with propensity to melt at a low temperature.

5 Three stem-loop variants of Trnc.A-30 containing uninterrupted stems with high G-C base pairs were therefore designed. In these variants the conserved sequence motif identified in Family I was unaltered (Table 5), but the stems had varying lengths. At 250 nM inhibitor concentration, Trnc.1-30 (SEQ ID NO:67) and Trnc.2-30 (SEQ ID NO:68) inhibited approximately 95%
10 of the activity of *Taq* polymerase, whereas Trnc.3-30 (SEQ ID NO:69) inhibited only about 60% of the polymerase activity (see below). Trnc.3-30 containing the shortest stem (7-base pairs) of the three variants was a poor inhibitor for *Taq* polymerase, indicating that additional contacts in the stem are required for productive interaction. To determine whether the decreased
15 inhibition observed with Trnc.3-30 is due to its reduced affinity to bind to the polymerase, the affinities of all three variants for binding to *Taq* polymerase were calculated. The K_d values fell between 2-3 nM (Table 5), indicating that all three variants had similar binding affinities. Hence, the lack of inhibition caused by Trnc.3-30 was not due to lack of binding, but presumably due to its
20 inability to block the active site. Affinities of the three variants for binding to *Taq* polymerase are about 75-fold lower than the full-length molecule (K_d of the full-length sequence is 40 pM), and about 3-5-fold lower than Trnc.A-30. The IC_{50} values for the three constructs decreased with the decrease in length of the stem; 25, 50 and 186 nM for Trnc.1-30, Trnc.2-30 & Trnc.3-30,
25 respectively (Figure 11). This result is in agreement with the notion that the ligands with longer stems are more effective inhibitors. The IC_{50} value of the full-length sequence is 22 nM. Hairpin extension assays were preformed at 30°C for 1 hour.

Although full length TQ30 inhibits *Tth* polymerase, neither Trnc.1-30

nor Trnc.2-30 inhibit *Tth* polymerase, despite the fact that the enzyme is completely inhibited by the full length ligand.

Stoffel fragment (61 kD) is a truncated form of *Taq* polymerase that lacks the 5'-3' exonuclease activity and is similar to 67 kD Klen*Taq* DNA polymerase (67 kD). The polymerase activity of the Stoffel fragment was completely inhibited by the full-length, as well as, the three truncated forms of TQ30. IC_{50} values of the three truncates are Trnc.1-30 = 2.7 nM, Trnc.2-30 = 5.9 nM and Trnc.3-30 = 10.3 nM (Figure 12). Overall, the three truncated forms of TQ30 are more effective in inhibiting the Stoffel fragment than *Taq* polymerase (compare Figure 11 with Figure 12). The IC_{50} values of these truncates for the inhibition of the Stoffel fragment are an order of magnitude better than those for *Taq* polymerase. The IT_{50} value for inhibition of the Stoffel fragment by Trnc.2-30 was 38°C (data not shown). Surprisingly, the TQ21 sequence, which inhibits both *Taq* and *Tth* polymerase does not inhibit the Stoffel fragment. This suggests that the binding site of TQ21 on the Stoffel fragment is either partially or completely deleted or has been reorganized upon truncation of the protein.

Truncates of ligand TQ21 (SEQ ID NO:59).

Unlike the Family I ligands, such as TQ30, the 30-nucleotide variable region of the Family II ligand, TQ21, does not inhibit either *Taq* or *Tth* polymerase (data not shown), indicating that the additional nucleotides from fixed regions are required for inhibition. Deletion analysis of the full-length TQ21 sequence led to the identification of a 51-mer sequence (Trnc.21 (SEQ ID NO:70) (Table 4)) that retained the ability to inhibit both *Taq* and *Tth* polymerases. In addition to the entire 30-nucleotide random region, the Trnc.21 sequence contained 9 and 12 nucleotides from the 5' and 3' fixed regions, respectively (Table 4). In contrast, to the TQ30 truncates, which showed decreased affinity for *Taq* polymerase, Trnc.21 showed increased affinity; the K_d of Trnc.21 for binding to *Taq* polymerase is 9 pM (Figure

13A), about 4-fold higher affinity than the full-length sequence. The IC_{50} value of Trnc.21 for inhibition of *Taq* polymerase is 21 nM (Figure 13B), about 3-fold lower than the value for the full-length sequence. The calculated IT_{50} values for *Taq* polymerase and *Tth* polymerase are 34°C and 35.6°C, respectively (Figure 13C). The hairpin extension assays were carried out between the temperatures of 35 and 50°C for 1 hour with 250 mM Trnc.21. Thus, based on the affinity and the values of IC_{50} and IT_{50} , the truncated form of TQ21 is a better inhibitor than the full-length sequence. Similar to the full-length sequence, Trnc.21 did not inhibit the activity of the Stoffel fragment.

Dimeric Forms of Truncates.

Multimerization of ligands increases effective local concentration, resulting in a longer resident time with the target (avidity). Based on its moderate affinity for *Taq* polymerase Trnc.2-30 was selected for synthesis of a homodimer (Table 4). Homodimer (D.30-D.30) (SEQ ID NO:71) (Table 4) of Trnc.2-30 was synthesized in tail-to-tail orientation (linked at 3' ends) using the symmetric dimer CPG as the support in solid phase chemical synthesis using standard methods.

The affinity of D.30-D.30 dimer for binding to *Taq* polymerase is 40 pM (Figure 14A), about 75-fold higher than its monomeric form. The IC_{50} value of the homodimer is 14 nM, about 3.5-fold lower than the monomeric form (Figure 14B). Thus, the dimerization of the truncated TQ30 produced a more effective inhibitor for *Taq* polymerase.

Two heterodimeric sequences in which the two monomeric truncates, Trnc.2-30 and Trnc-21 (Table 4), were joined by a linker containing 3 thymines were also prepared. In D.21-D.30 (SEQ ID NO:72) the Trnc-21 sequence is placed at the 5' end of the molecule, whereas in D.30-D.21 (SEQ ID NO:73) it occupies the 3' end of the molecule. Unlike the full-length TQ30, its truncated forms did not inhibit *Tth* polymerases. Trnc-2, on the other hand,

inhibited both *Taq* and *Tth* polymerases, but not the Stoffel fragment. Assuming that the monomeric units are able to function independently, after being tethered into a single sequence, the combination of the two truncated ligands would provide a single sequence that could inhibit all three
5 polymerases. At the lowest inhibitor concentration (62.5 nM) the inhibitory effect of the two heterodimers on *Taq* polymerase is higher than the two monomers. The effect of heterodimers on *Tth* polymerase is identical to that of the Trnc-21 monomer. The Stoffel fragment could not completely extend the hairpin template in the presence of the two heterodimers. In contrast, partially
10 extended products were less abundant in the presence of the monomeric Trnc.2-30 sequence. The lack of the complete extension of the hairpin template suggests that the heterodimers do suppress the activity of the Stoffel fragment.

The heterodimer D.21-D.30 has an IC_{50} value of approximately 30 nM
15 for the inhibition of the *Taq* and *Tth* polymerases (Figure 15A). The IT_{50} values for the inhibition of the *Taq* and *Tth* polymerase are 41 and 34.5°C, respectively (Figure 15B). D.21-D.30 inhibits the Stoffel fragment with an IC_{50} value of 15.5 nM and an IT_{50} value of 38°C (data not shown). The K_d of ligand D.21-D.30 heterodimer for binding to *Taq* polymerase is similar to that
20 of the Trnc-21 (10 pM), suggesting that the protein preferentially binds to the sequence motif with high-affinity binding.

The positioning of the two monomeric units in the dimer seems to have no overall effect on the inhibition on any of the three polymerases. The two different monomeric units did not show adverse effect when they were
25 combined into a dimer. As expected, the heterodimers showed the ability to inhibit all three polymerases quite effectively, indicating that by and large, functions of monomeric units in heterodimers are mutually exclusive.

The following Examples are provided to explain and illustrate the present invention and are not intended to be limiting of the invention.

Example 1. Experimental Procedures

A. Materials and Methods.

Recombinant *Taq* polymerase (r*Taq*; Mr 94 kDa) suspended in a buffer consisting of 100 mM KCl, 20 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 50% glycerol (v/v) and 0.2% Tween 20 and recombinant *Tth* polymerase (r*Tth* Mr 94 kDa) suspended in a buffer consisting of 50 mM Bicine-KOH (pH 8.3), 90 mM KCl and 50% glycerol (v/v) were purchased from Roche Molecular Systems, Inc. (Alameda, CA). *Taq*, *Tth* and *Ultma* DNA polymerases were obtained from Perkin Elmer. *Ultma* polymerase is a deleted form of *Tma* polymerase that lacks the wild type 5'-3' exonuclease activity. *Tli* and *Tfl* DNA polymerases were purchased from Promega. *Tbr* polymerase (Thermalase Tbr) was obtained from Amresco Inc. Symmetrical branching 3'-3' linking CPG and C-6 Thiolmodifier phosphoramidites were obtained from Clontech (Palo Alto, CA). ULTRALINK™ Iodoacetyl beads were purchased from Pierce Chemicals (Rockford, IL). Enzymes used in radiolabeling of DNA were obtained from Boehringer Mannheim (Indianapolis, IN). All other reagents and chemicals were analytical grade and purchased from standard commercial sources.

Preparation of Oligonucleotides.

Oligonucleotides were synthesized by standard solid phase cyanoethyl phosphoramidite chemistry and purified by denaturing polyacrylamide gel electrophoresis to size homogeneity before use. The symmetrical homodimer was synthesized with Symmetrical Branching 3'-3' linking CPG. DNA concentrations were based on $33 \mu\text{g/mL} = 1 A_{260} \text{ Unit}$.

Preparation of Affinity Beads.

Twenty five nanomoles of either ligand TQ21 (SEQ ID NO:59) or TQ30 (SEQ ID NO:50) (Table 3) containing a thiol group at the 5' end was deprotected with AgNO_3 and dithiothreitol (DTT) according to Manufacturer's instructions. Excess DTT was removed by four sequential extractions with

equal volumes of ethyl acetate. The deprotected ligand was then mixed with 500 μ L of ULTRALINK™ iodoacetyl beads that had been washed two times in a buffer consisting of 50 mM Tris-HCl (pH 8.3) and 5 mM EDTA. The reaction mixture was incubated at room temperature for 2 hours on a rotating platform. Unreacted sites on the iodoacetyl beads were capped by reacting the mixture with 50 μ L of a 0.5 M cysteine solution in the same buffer for 15 minutes. Control beads were prepared by reacting 500 μ L of iodoacetyl beads with 500 μ L of 0.5 M cysteine. After the reaction, the beads were washed five times with 500 μ L of a PCR buffer consisting of 75 μ M heparin, 12.5 mM $MgCl_2$, 50 mM KCl and 10 mM Tris-HCl (pH 8.3).

B. SELEX.

The SELEX procedure has been described in detail in United States Patent No. 5,270,163. The SELEX experiments on both polymerases were performed using the template and primers shown in Table 1. The selection on *Taq* polymerase was carried out at room temperature in a buffer consisting of 10 mM Tris-HCl (pH 8.3; at 22°C), 50 mM KCl and 2.5 mM $MgCl_2$ (*Taq* binding buffer). The selection on *Tth* polymerase was carried out in a buffer containing 50 mM Bicine-KOH (pH 8.3; at 25°C), 90 mM KCl and 3.5 mM $Mn(OAc)_2$ (*Tth* binding buffer).

Each SELEX experiment was initiated with 5 nmoles of synthetic, gel-purified random sequence pool single stranded DNA (ssDNA) consisting of 30 nucleotide randomized region, flanked by 5' and 3' regions of fixed structure (Table 1). In a typical round of selection, ssDNA suspended in the appropriate binding buffer was heated to 90°C for 3 minutes, chilled on ice, and then brought to room temperature. Once equilibrated at room temperature, the DNA was incubated for 15 minutes with the appropriate target polymerase in the presence of 2 nmoles of tRNA as a competitor and 0.01% human serum albumin (hSA). Polymerase-DNA complexes were separated from unbound

DNA by nitrocellulose filtration through a prewet nitrocellulose filter (0.45 μ M, Millipore) under suction. The filter was immediately washed with 20 mL of the binding buffer, 20 mL of 0.5 M urea in the binding buffer, and 0.5 M urea in water. Filter retained DNA was eluted and isolated by ethanol
5 precipitation in the presence of carrier tRNA (5 μ g).

The isolated DNA was amplified by PCR with Primer Set I (Table 1). One of the primer strands contained three contiguous biotins at the 5' end. The unbiotinylated strand of the resulting duplex DNA was isolated by gel electrophoresis under denaturing conditions (Pagratis *et al.* in preparation) and
10 used for the next round of selection. In subsequent rounds, prior to incubating with the target polymerase, DNA pools were passed through nitrocellulose filters (counter selection) to remove DNA sequences that bind to the nitrocellulose filter. The number of picomoles of target polymerase was gradually decreased during the course of SELEX to increase the selective
15 pressure for sequences with high affinity binding. The amount of DNA in each selection was kept at least five-fold higher than the amount of protein to ensure competition for high affinity binding DNA sequences.

The progress of SELEX was monitored by nitrocellulose filter binding analysis of enriched pools. The enriched pools that showed the highest affinity
20 binding were PCR amplified with Primer Set II to incorporate BamHI and EcoRI restriction sites at the termini of the resulting duplex DNA. This DNA was gel purified and digested with BamHI and EcoRI and cloned into plasmid pUC18 vector previously digested with the same enzymes using standard techniques. (Sambrook *et al.* (1989) in Molecular Cloning: A laboratory
25 Manual, 2nd ed., Part 3, pC.1, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). Clones were isolated and sequenced by standard dideoxy sequencing technique (Sequenase kit from U.S. Biochemical, Cleveland, OH).

C. Nitrocellulose Filter Binding Assay

For isolation of DNA molecules that bind tightly to *Taq* polymerase and *Tth* polymerase, respectively, the nitrocellulose filter partitioning method was used as described in the SELEX Patent Applications. Briefly, gel-purified ³²P ss-DNA pools labeled at the 5' end were suspended in the binding buffer, heated to 80°C, chilled on ice and then brought to room temperature. The DNA (5-10 pM) was then incubated for 15 minutes at room temperature with varying amounts of the target polymerase in 50 µL of the appropriate binding buffer containing 0.1 µg of tRNA and 0.01% hSA. The DNA concentrations were kept lower than 100 pM to ensure equilibrium in the presence of excess protein concentrations. After 15 minutes the binding reaction mixtures were passed through pre-wet nitrocellulose/cellulose acetate mixed matrix filters (0.45 µm pore size, Millipore Corporation, Bedford, MA) and the filters were immediately washed with 5 mL of binding buffer. The amount of DNA bound to the filters was quantitated by measuring the radioactivity of the filters by liquid scintillation counting. The quantity of DNA bound to filters in the absence of protein was used for background correction. The percentage of input DNA retained on each filter was plotted against the corresponding log of the polymerase concentration (Figures 1 and 2). The nonlinear least square method was used to obtain the dissociation constants (K_d) of the DNA ligands to the *Taq* and *Tth* polymerases, respectively. (Schneider *et al.* (1995) Biochemistry 34:9599; Jellinek *et al.* (1993) Proc. Natl. Acad. Sci., U.S.A. 90:11227-11231).

The unselected random sequence pool bind *Tth* polymerase with an estimated K_d of approximately 70 nM (Figure 1B, (•)), whereas the K_d of this pool binding to *Taq* polymerase is approximately 50-100 nM (Figure 1A, (◊)). After 12 rounds of selection, the K_d of binding to *Taq* polymerase was 3.5 nM (Figure 1A, (◊)). Additional rounds of selection did not result in further improvement of affinity. Thus, the resulting affinity of the enriched pool to

Taq polymerase was significantly improved as compared to the unselected random pool. Similar results were obtained with the *Tth* polymerase where the pool from the 10th round showed a K_d of 5 nM (Figure 1B, (o)).

The ssDNA pool selected for *Taq* polymerase showed very tight
5 binding to *Tth* polymerase with a K_d of 0.2 nM (Figure 2A, (o)). This result is not surprising, since the amino acid sequence identity between the two polymerases is approximately 87% (Asakura *et al.* (1993) J. Ferment. Bioeng. 76:265-269). The pool selected for *Tth* polymerase bound *Taq* polymerase in a different manner, with the binding saturating at around the 50% level (Figure
10 2B, (o)), suggesting that about one half of the sequences in the pool are not interacting with *Taq* polymerase. Based on 50% saturation the estimated K_d is 0.3 nM.

The ss-DNA sequences obtained from 10 rounds of selection performed with *Tth* polymerase are set forth in Table 2. Twenty nine individual clones
15 were sequenced from the *Tth* polymerase selection (only the variable 30 nucleotide region is shown in Table 2). The sequences were grouped into two families based upon sequence similarity. The ss-DNA sequences obtained from 12 rounds of selection performed with *Taq* polymerase are set forth in Table 3. Thirty three unique sequences were isolated. The lowercase letters in
20 some of the sequences depict the 5'-fixed sequence and the upper case letters depict the 30 nucleotide random region. The sequences were grouped into three families based on sequence similarity.

Example 2. Polymerase Inhibition Assays

25 The polymerase inhibition assays were performed using the template DNA (DNA-HP; 5'-ATGCCTAAGTTTCGAACGCGGCTAG CCAGCTTTTGCTGGCTAGCCGCGT-3' (SEQ ID NO:6)), end-labeled at the 5' end with T4 polynucleotide kinase and ^{32}P - γ -ATP and purified by gel electrophoresis under denaturing conditions (Figure 4). In a representative

experimental procedure, either 0.25 pmoles of *Taq* polymerase (5 U) or 0.125 pmoles (2.5U) of *Tth* polymerase was mixed with 5 pmoles (250 nM) of the enriched pool, random pool or a specific DNA ligand in the standard PCR buffer (20 μ L). Five pmoles (250 nM) of labeled template DNA-HP was
5 added and the mixture was incubated at different temperatures for a given period of time. The reaction was stopped by adding EDTA to a final concentration of 125 mM (5 μ L of 0.5 M EDTA). The DNA was resolved on a polyacrylamide gel under denaturing conditions. Gels were visualized by autoradiography and the percent DNA bound was quantitated by
10 phosphoimager. Variations in this general procedure for specific reactions are noted in the Specification.

The order in which the oligonucleotide inhibitors are added to the reaction mixture is irrelevant, as long as, the template is added last. The oligonucleotides require Mg^{++} ions, an essential component of PCR, to function
15 and appear to tolerate many buffer systems.

Figure 5 illustrates the results of the polymerase activity assays using the enriched pools of DNA. Figures 6-9 illustrate the results of the polymerase activity assays using ligands TQ30 (SEQ ID NO:50) and TQ21 (SEQ ID NO:59).

20

Measurement of IC_{50} Values.

IC_{50} values (the concentration of inhibitor required to produce 50% of the product in the assay) were obtained by using hairpin extension assay. In a typical inhibition assay, a 20 μ L reaction contained either 0.25 pmoles of *Taq*
25 polymerase (5 U) or 0.125 pmoles of *Tth* polymerase (2.5 U), oligonucleotide inhibitor (at varying concentrations), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, and 1 mM each dNTPs. Gel purified, 5'-end-labeled hairpin DNA substrate (DNA-HP; 5'-ATGCCTAAGTTTCGAACGCGGCT
AGCCAGCTTTTGCTGGCTAGCCGCGT-3') was then added to a final

concentration of 250 nM and the reaction was incubated at 30°C for 1 hour. The reaction was stopped by adding 5 μ L of 0.5 M EDTA (pH 8.0) followed by formamide gel loading buffer. Extension products were resolved on 10% polyacrylamide gels under denaturing conditions. The amount of extension products were quantitated by phosphorimager. The amounts of products formed in the presence of inhibitor was normalized to the product formed in the absence of an inhibitor to obtain the percent of product.

Measurements of IT_{50} Values.

Hairpin extension reactions were the same as described above, except that the inhibitor concentration was 250 nM. Incubation time at each temperature was 1 hour. The amount of product was quantitated by phosphorimager and normalized to the product formed in the absence of an inhibitor at the same temperature to obtain the percent of product.

Determination of Ligand TQ30 and Ligand TQ21 Substrate Activity.

In a representative experimental procedure 5'-end labeled ligand TQ30 (SEQ ID NO:50), TQ21 or TQ21 (3'-capped with an ethylene glycol linker) (approximately 3 pmole) was incubated in 20 μ L of the binding buffer and 1 mM each dNTPs in the absence and presence of either 5 U of *Taq* polymerase or 2.5 U of *Tth* polymerase for 16 hours at room temperature. Capping of the 3'-end of TQ21 was accomplished with an ethylene glycol linker (3'-Spacer C3 support from Glen Research) using standard conditions known in the art.

Affinity Capture Assays.

The affinity capture reactions were performed at 70°C for 5 minutes in a 100 μ L reaction volume that contained: 75 μ M heparin, 12.5 mM $MgCl_2$, 1 mM each dNTPs, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 5 U of *Taq* polymerase or 2.5 U of *Tth* polymerase and 250 nM 5'-end labeled hairpin

- assay template (DNA-HP). After 5 minutes the reaction mixture was diluted by three fold and cooled to 4°C. After round 1 synthesis, 15 µL of beads (either affinity beads or control beads, prepared as described above) were added to the reaction mixture at 4°C and gently mixed for 10 minutes.
- 5 Supernatants containing the labeled template were recovered after centrifugation and saved for gel analysis. The beads were then washed five times with 100 µL of a buffer consisting of 75 µM heparin, 12.5 mM MgCl₂, 50 mM KCl and 10 mM Tris-HCl (pH 8.3). After round 2 synthesis, the washed beads were mixed with a fresh aliquot of the reaction mixture
- 10 containing all of the reagents except the polymerase. After incubating at 70°C for 5 minutes, the reaction mixture was recovered and analyzed by gel electrophoresis.

Example 3. Exonuclease Inhibition Assay

- 15 The exonuclease inhibition assays were performed using the designed template,
- 5'-TTCGAGCGTGAATCTGAATTCGCGGCTAGCCAGCTTTTGCTG
GCTAGCCGCGGTGGGAACTGAGGTAGGTGTTTTCACCTACCTCAG
TTTCCCACC-3' (Exo-sub) (SEQ ID NO:75), radiolabeled at the 5' end (using
- 20 [γ ³²P]-ATP and T4 polynucleotide kinase) and at the 3' end using ([α ³²P]-ddATP and deoxyterminaltransferase). In a representative experimental procedure either 5 U of *Taq* polymerase or 2.5 U of *Tth* polymerase was mixed with 250 nM of ligand TQ30 or ligand TQ21 in the standard PCR buffer (20 µL), followed by the addition of the double-labeled Exo-Sub (250 nM, added
- 25 last). After incubating for 16 hours at room temperature, the reactions were quenched by addition of EDTA to 0.1 mM final concentration. Cleavage products were resolved on 8% polyacrylamide gels run under denaturing conditions.

Example 4. Polymerase Inhibition Assays

Inhibition by TQ21 (SEQ ID NO:50) and TQ30 (SEQ ID NO:50) was tested on (A) thermophilic DNA polymerases, (B) mesophilic DNAPs (*Taq* polymerase as a control), and reverse transcriptases, and (C) RTs. All reactions were carried out in 20 μ L volume with the HP hairpin template (Example 2) in the presence of 1 mM each dNTPs, using either 250 or 500 nM of ligand TQ21 or TQ30. Specific reaction conditions for each polymerase were as follows:

Thermostable polymerases: *Tma* polymerase: *Uitma* polymerase (6 U), 10 mM Tris-HCl, pH 8.8, 10 mM KCl, 2.5 mM MgCl₂ and 0.002% Tween 20 (v/v); *Tbr* polymerase (2U), 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5 mM MgCl₂ and 0.01% Triton X-100; *Tti* polymerase (3U) and *Tfi* polymerase (5 U), 10 mM Tris-HCl; pH 9.0, 50 mM KCl and 0.1% Triton X-100.

Mesophilic polymerases: All incubations including *Taq* polymerase (5U) (an internal control for the buffer) were performed in a buffer consisting of 10 mM Tris-HCl, pH 7.5, 40 mM KCl, 5 mM MgCl₂ and 7.5 mM DTT (Klenow fragment (5U); T4 DNA polymerase (4U); T7 DNA polymerase (7U)).

Reverse Transcriptases. All incubations were performed in a buffer consisting of 50 mM Tris-HCl, pH 8.3, 60 mM NaCl, 6 mM Mg(OAc)₂ and 10 mM DTT. (HIV-1 RT (0.56 pmoles); AMV RT (1U); M-MLV RT (10 U); Superscript II (Sscript II) (10 U).

Example 5. Detection of Low Copy Number Target

PCR amplifications were performed using a system that amplifies a 203-bp target-specific product from HIV-2 LTR as described by Respass *et al.* (1994) in Interscience Conference on Antimicrobial Agents and Chemotherapy 94:110). All PCR amplifications were carried out in the presence of 1.3 μ g of human placental DNA, 0.4 mM each dNTP, 25 pmoles of each primer, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl₂, 10% glycerol, 5 U of *Taq* polymerase and

- the template (approximate number of copies as indicated in Figures 10A-10C) in 100 μ L reaction volume. Thermocycling was performed at 50°C for 2 minutes followed by 94°C for 30 sec; 60°C for 30 seconds; 72°C for 30 seconds and then autoextended 60°C annealing in 1°C increments for 5 cycles.
- 5 This followed a 35-cycle amplification at 90°C for 30 seconds; 65°C for 30 seconds; 72°C for 30 seconds.

"Hot start" PCR was performed by using "AmpliWax" beads (from Perkin Elmer) according to manufacture's instructions. All other PCR amplifications were carried out without "hot start" conditions.

- 10 "NeXstart" PCR was performed using ligands TQ30 and TQ21, (50 nM final concentration) as inhibitors. One amplification was performed in the presence of a nonspecific oligonucleotide (50 nM final concentration) for purposes of comparison.

Table 1

Starting Random Sequence Pool of ssDNA:

5' -TTCTCGGTTGGTCTCTGGCGGAGC- [N]₃₀-TCTTGTGTATGATTCGCTTTTCCC-3'
(SEQ ID NO:1)

SELEX PCR Primer Set I:

5' -TTCTCGGTTGGTCTCTGGCGGAGC-3' (SEQ ID NO:2)
(SEQ ID NO:2)

5' -BBBTAGGGAAAAGCGAATCATACACAAGA-3' (SEQ ID NO:3)
(SEQ ID NO:3)

(B represents Biotin)

SELEX PCR Primer Set II:

5' -GGCGAATTCCTTCTCGGTTGGTCTCTGGCGGAGC-3'
EcoRI (SEQ ID NO:4)

5' -CGCGGATCCTAATACGACTCACTATAGGGAAAAGCGAATCATACACAAGA-3'
BamHI (SEQ ID NO:5)

Table 2

SEQ
ID CLONE
NO: NO:

SEQUENCE (5'-3')

CLASS I

7	2:	TATCGTTTACTCATT	GTTTTG	TGTGT
8	34:	ACATTACCCGAGACATTCC	TGAC	GTTTTG
9	21:	TGCTGCTCCTTGTTT	C	GTTTTG TCT
10	18:	AGCTTTGGGGACATTCTAAC	GTTTTG	TCA
11	19:	AGATGCTTCA	GTTTTT	TCTCCGTG
12	16:	T	CTTTTTG	GA
13	12:	ATGGTC	TTTTTG	TTGTTTGTTTG
14	9:	GTGA	CTTTTT	ACTTGTCCTAGGCTG
15	15:	CATCTAT	GTCCTC	TTTATATTTGG
16	14:	ACTACCTGG	TTGTGTG	CTTTCCAT
17	25:	ATCCATGAGACTAG	GTTGGT	TAGGGTGGTG
18	1:	CCCTCATA	GTTTAA	CTTTACCTGGCTTATC
19	10:	AGTGAACACCTTCT	GTTTCG	TGAGTC
20	23:	CGTGT	GTCTTA	GTTAGCTCGTGG
21	24:	TAACGTTGTGT	GTTCTG	TGCTA
22	26:	AACAGATTGGTCATAT	TCCTTG	G
23	27:	TGTGTTAT	GCTCCG	GTAACAATGCCCTT
24	30:	AATTGTA	ATTTCC	GTATCTCTG
25	33:	GCA	ATTTCC	TGTCCAATCATTGTAG
26	36:	GCTTGAA	GCTTTC	ACCCATCCTA/GA
27	41:	CTTCTCCTTTATAT	GTCTTA	CCA
28	42:	TATCGAGTAGACCCTGTT	GTTTCG	G
29	44:	CGC	GTCTAG	CTAAGATTTCTACTGATGCAT
30	46:	ATG	ATTTTA	TGTTTATCCTGTTT

SEQ
ID CLONE
NO: NO:

SEQUENCE (5'-3')

CLASS II

31	45:	CAGTCGCTGTACGTGCTCTCCCTATGTAAC
32	6:	CAATCGGTGTACAATATCTTCC
33	28:	CGTTAGCTGGTTAGTTAGTACTAG
34	35:	AGGTAAGCGATTATGGGGTTATCG
35	40:	TAGTTACATGAACTAATCGTGGAG

Table 3

SEQ

ID CLONE:

NO: NO:

SEQUENCE (5'-3')

Family I

36	12:	(4)	ggcggagc	GATGTACAGTATC	GCTATCGAAAGAGGCTG
37	15:		ggcggagc	AGTGTGCAGTAGT	GTGATGTCAGAGTATCC
38	18:		ggcggagc	AGTGTGCGGTAGT	GTGATCTGAGAGTATCC
39	26:		ggcggagc	AGTGTGTAGTAGT	GTTACGATGGGGACGG
40	40:		ggcggagc	AGTGTACAGTAGT	GTTCCCGGTAGAGCTAT
41	27:		ggcggagc	AATGTGCAGTATT	GATATCGCTGGTGGTCA
42	10:	(2)	ggcggagcA	AGTGTACAGTAGT	TGCCTACGCTAGTG
43	6:		ggcggagcA	AGTGTGCAGTAGT	TACTCATAAGAGACCA
44	34:		ggcggagcA	AGTGTACAGTAGT	TGCCTACGCTAGTG
45	28:		ggcggagcAC	AATGTGAAGTATT	GGGGTACGTCAGTAG
46	5:		CAAGCGGAAAC	AATGTACAGTATT	GGGATC
47	33:		AAGGCCATT	GATGTACAGTATC	AATGCTGC
48	29:		AATTGGGAAAC	AATGTGCAGTAGT	TGAAGG
49	44:		AAATGGGAAAC	AATGTGCAGTATT	GGAAGG
50	30:	(3)	AAGACCAGAC	AATGTACAGTATT	GGCCTGA
76	3:		TCAATACACAAATT	GATGTACAGTGTC	GAT

Family II

51	42:		TACGCTGACAGGCC	ACGTTTTG	TCATGAT
52	22:		GAGAACTCCGTTCTTA	GCGTATTG	GAGTCC
53	2:		AGGTGGGACATTCTTT	GCGTTATG	TCTCTGA
54	49:		GGGCTCGGAACATTCTTA	GCGTTTTG	TTCC
55	50:		ATAGGCAGGGGACATTGCA	ACCTTTTG	TCA
56	7:		AATTGAAGTGACTTTCTCT	GCGTTTAG	TCG
57	39:		AGGAATCTGGGGCATTCTTT	GCGTTTTG	CG
58	41:		CTCAGGATAAGGTCATTCTA	ACGTTATG	A
59	21:		GATCATCTCAGAGCATTCTTA	GCGTTTTG	T
60	31:		GATCATCTAAGAGCATTCTTA	GCGTTTTG	G
61	43:		CAAAACGAGAGAGCTTTCTGT	GCGTTTAG	C
62	23:		GACCAAGCGTCAAGATATTCAA	ACGTTTTA	
63	25:		AGAAGCATAACGAAGACATTCCA	ACGTTTGG	
64	9:	(2)	AATCGATTGTTGAACATTCTG	ACGTTTTG	T
65	17:	(2)	AGAAGCATAACGAAGACATTCCA	ACGTTTTG	
66	36:		AGAAGCATAACGAAGACATTCCA	ACGTTTTG	

Family III

77	4:	(2)	CATTGGGCCAGAGGAACACAACCTCAACAG		
----	----	-----	--------------------------------	--	--

Table 4

SEQ ID NO:	CLONE NO:	SEQUENCE
50	TQ30	5'-ttctcgggtgggtctctggtggagcagaccagacaatgtacagtattggcctga tcttgatgatgattcgcttttccc-3'
59	TQ21	5'-ttctcgggtgggtctctggtggagcgatcatctcagagcattcttagcggttttgt tcttgatgatgattcgcttttccc-3'
67	Trnc. 1-30	5'-GGGACCAGACAATGTACAGTATTGTCTGGTCCC-3'
68	Trnc. 2-30	5'-GCCGGCCAATGTACAGTATTGGCCGGC-3'
69	Trnc. 3-30	5'-GGCCAATGTACAGTATTGGCC-3'
70	Trnc-21	5'-tggtggagcgatcatctcagagcattcttagcggttttgttcttgatga-3'
71	D.30-D.30	5'-GCCGGCCAATGTACAGTATTGGCCGGC 5'-GCCGGCCAATGTACAGTATTGGCCGGC ^x
72	D.21-D.30	5'-tggtggagcgatcatctcagagcattcttagcggttttgttcttgatgat ^T 3'-CGGCCGGTTATGACATGTAAACCGCCGT ^T
73	D.30-D.21	5'-GCCGGCCAATGTACAGTATTGGCCGGC ^T 3'-agtatgtgtcttggttttgcgattcttaccgagacactactagcaggcggtt ^T

Table 5

SEQ ID NO:	CLONE NO:	Secondary Structure ¹	K _d (nM)	T _m (°C)
74	Trnc.A-30 (30-mer)	<p>5'-AAGAC A CAG CAATGT A</p> <p>3'-A CG GTC GTTATG A</p>	0.6±0.1	51
67	Trnc.1-30 (33-mer)	<p>5'-GGGACCAGAGGCCAATGT A</p> <p>3'-CCCTGGTCTCCGTTATG A</p>	2.0±0.3	77
68	Trnc.2-30 (27-mer)	<p>5'-GCCGGCCCAATGT A</p> <p>3'-CGGCCCGTTATG A</p>	3.1±0.3	81.5

Table 5 (Continued)

SEQUENCE LISTING

- (1) GENERAL INFORMATION:
- (i) APPLICANT: LARRY GOLD
SUMEDHA JAYASENA
 - (ii) TITLE OF THE INVENTION: NUCLEIC ACID LIGAND INHIBITORS
TO DNA POLYMERASES
 - (iii) NUMBER OF SEQUENCES: 77
 - (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Swanson and Bratschun, L.L.C.
 - (B) STREET: 8400 East Prentice Ave., Suite 200
 - (C) CITY: Denver
 - (D) STATE: Colorado
 - (E) COUNTRY: USA
 - (F) ZIP: 80111
 - (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Diskette, 3.5 inch, 1.44 Mb
storage
 - (B) COMPUTER: IBM Compatible
 - (C) OPERATING SYSTEM: MS-DOS
 - (D) SOFTWARE: WordPerfect 6.0
 - (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: PCT/US96/_____
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
 - (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 08/487,426
 - (B) FILING DATE: 7-JUNE-1995
 - (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 08/487,720
 - (B) FILING DATE: 7-JUNE-1995
 - (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 08/484,557
 - (B) FILING DATE: 7-JUNE-1995
 - (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Barry J. Swanson
 - (B) REGISTRATION NUMBER: 33,215
 - (C) REFERENCE/DOCKET NUMBER: NEX43C/PCT
 - (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (303) 793-3333
 - (B) TELEFAX: (303) 793-3433
- (2) INFORMATION FOR SEQ ID NO:1:
- (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 78 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

56

- (ii) MOLECULAR TYPE: DNA
- (ix) FEATURE:
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TTCTCGGTTG GTCTCTGGCG GAGCNNNNNNN NNNNNNNNNN NNNNNNNNNN 50
NNNNTCTTGT GTATGATTCG CTTTCCCC 78

- (2) INFORMATION FOR SEQ ID NO:2:
 - (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TTCTCGGTTG GTCTCTGGCG GAGC 24

- (2) INFORMATION FOR SEQ ID NO:3:
 - (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (ix) FEATURE:
 - (D) OTHER INFORMATION: N at positions 1, 2, and 3 is biotin
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

NNNTAGGGAA AAGCGAATCA TACACAAGA 29

- (2) INFORMATION FOR SEQ ID NO:4:
 - (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGCGAATTCT TCTCGGTTGG TCTCTGGCGG AGC 33

- (2) INFORMATION FOR SEQ ID NO:5:
 - (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 50 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGCGGATCCT AATACGACTC ACTATAGGGA AAAGCGAATC ATACACAAGA 50

- (2) INFORMATION FOR SEQ ID NO:6:
 - (i) SEQUENCE CHARACTERIZATION:

57

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATGCCTAAGT TTCGAACGCG GCTAGCCAGC TTTTGCTGGC TAGCCGCGT 49

- (2) INFORMATION FOR SEQ ID NO:7:
 - (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 74 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TTCTCGGTTG GTCTCTGGCG GAGCTATCGT TTAATCATTG TTTGTGTGT 50
TCTTGTGTAT GATTCGCTTT TCCC 74

- (2) INFORMATION FOR SEQ ID NO:8:
 - (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 76 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TCTCGGTTGG TCTCTGGCGG AGCACATTAC CCGAGACATT CCGACGTTT 50
TGTCTGTGTG ATGATTCGCT TTTCCC 76

- (2) INFORMATION FOR SEQ ID NO:9:
 - (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 72 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TTCTCGGTTG GTCTCTGGCG GAGCTGCTGC TCCTGTTCG TTTGTCTTC 50
TTGTGTATGA TTCGCTTTTC CC 72

- (2) INFORMATION FOR SEQ ID NO:10:
 - (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 78 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TTCTCGGTTG GTCTCTGGCG GAGCAGCTTT TGGGGACATT CTAACGTTTT 50

GTCATCTTGT GTATGATTCG CTTTCC

78

- (2) INFORMATION FOR SEQ ID NO:11:
- (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 72 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TTCTCGGTTG GTCTCTGGCG GAGCAGATGC TTCAGTTTTC TCTCCGTGTC
TTGTGTATGA TTCGCTTTC CC

50

72

- (2) INFORMATION FOR SEQ ID NO:12:
- (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 73 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TTCTCGGTTG GTCTCTGGCG GAGCTCTTTT GGACTGAAGG TTGTGTTGGTT
CTTGTGTATG ATTCGCTTTT CCC

50

73

- (2) INFORMATION FOR SEQ ID NO:13:
- (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 71 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TTCTCGGTTG GTCTCTGGCG GAGCATGGTC TTTTGTGTGT TTGTTGTCT
TGTGTATGAT TCGCTTTTCC C

50

71

- (2) INFORMATION FOR SEQ ID NO:14:
- (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 73 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TTCTCGGTTG GTCTCTGGCG GAGCGTGA CT TTTACTTGT CCTAGGCTGT
CTTGTGTATG ATTCGCTTTT CCC

50

73

- (2) INFORMATION FOR SEQ ID NO:15:
- (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 72 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single

- (D) TOPOLOGY: linear
(ii) MOLECULAR TYPE: DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TTCTCGGTTG GTCTCTGGCG GAGCCATCTA TGTCTTCTTT ATATTGGTC 50
TTGTGTATGA TTCGCTTTTC CC 72

- (2) INFORMATION FOR SEQ ID NO:16:
(i) SEQUENCE CHARACTERIZATION:
(A) LENGTH: 72 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULAR TYPE: DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TTCTCGGTTG GTCTCTGGCG GAGCACTACC TGGTTGTGTG CTTTCCATTC 50
TTGTGTATGA TTCGCTTTTC CC 72

- (2) INFORMATION FOR SEQ ID NO:17:
(i) SEQUENCE CHARACTERIZATION:
(A) LENGTH: 78 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULAR TYPE: DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TTCTCGGTTG GTCTCTGGCG GAGCATCCAT GAGACTAGGT TGGTTAGGGT 50
GGTGTCTTGT GTATGATTCG CTTTCCCC 78

- (2) INFORMATION FOR SEQ ID NO:18:
(i) SEQUENCE CHARACTERIZATION:
(A) LENGTH: 78 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULAR TYPE: DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TTCTCGGTTG GTCTCTGGCG GAGCCCCCTCA TAGTTTAACT TTACCTGGCT 50
TATCTCTTGT GTATGATTCG CTTTCCCC 78

- (2) INFORMATION FOR SEQ ID NO:19:
(i) SEQUENCE CHARACTERIZATION:
(A) LENGTH: 74 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULAR TYPE: DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TTCTCGGTTG GTCTCTGGCG GAGCAGTGAA CACCTTCTGT TTCGTGAGTC 50
TCTTGTGTAT GATTCGCTTT TCCC 74

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERIZATION:

- (A) LENGTH: 71 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TTCTCGGTTG GTCTCTGGCG GAGCCGTGTG TCTTAGTTAG CTCGTGGTCT 50
TGTGTATGAT TCGCTTTTCC C 71

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERIZATION:

- (A) LENGTH: 70 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TTCTCGGTTG GTCTCTGGCG GAGCTAACGT TGTGTGTTCT GTGCTATCTT 50
GTGTATGATT CGCTTTTCCC 71

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERIZATION:

- (A) LENGTH: 72 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

TTCTCGGTTG GTCTCTGGCG GAGCAACAGA TTTGGTCATA TTCCTTGGTC 50
TTGTGTATGA TTCGCTTTTC CC 72

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERIZATION:

- (A) LENGTH: 76 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TTCTCGGTTG GTCTCTGGCG GAGCTGTGTT ATGCTCCGGT AACAAATGCCC 50
TTTCTTGTGT ATGATTCGCT TTTCCC 76

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERIZATION:

- (A) LENGTH: 70 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TTCTCGGTTG GTCTCTGGCG GAGCAATTGT AATTTCGGTA TCTCTGTCTT 50
GTGTATGATT CGCTTTTCCC 70

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERIZATION:

- (A) LENGTH: 73 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TTCTCGGTTG GTCTCTGGCG GAGCGCAATT TCCTGTCCAA TCATTGTAGT 50
CTTGTGTATG ATTCGCTTTT CCC 73

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERIZATION:

- (A) LENGTH: 72 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

TTCTCGGTTG GTCTCTGGCG GAGCGCTTGA AGCTTTCACC CATCCTRATC 50
TTGTGTATGA TTCGCTTTTC CC 72

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERIZATION:

- (A) LENGTH: 71 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TTCTCGGTTG GTCTCTGGCG GAGCCTTCTC CTTTATATGT CTTACCATCT 50
TGTTGTATGAT TCGCTTTTCC C 71

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERIZATION:

- (A) LENGTH: 73 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

TTCTCGGTTG GTCTCTGGCG GAGCTATCGA GTAGACCCTG TTGTTCTGT 50
CTTGTGTATG ATTCGCTTTT CCC 73

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERIZATION:

62

- (A) LENGTH: 78 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

TTCTCGGTTG GTCTCTGGCG GAGCCGCGTC TAGCTAAGAT TTCTACTGAT 50
GCATTCTTGT GTATGATTCG CTTTTCCT 78

- (2) INFORMATION FOR SEQ ID NO:30:
 - (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 71 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

TTCTCGGTTG GTCTCTGGCG GAGCATGATT TTATGTTTAT CCTGTTTTCT 50
TGTGTATGAT TCGCTTTTCC C 71

- (2) INFORMATION FOR SEQ ID NO:31:
 - (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 78 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

TTCTCGGTTG GTCTCTGGCG GAGCCAGTCG CTGTACGTGC TCTCCCTATG 50
TAACTCTTGT GTATGATTCG CTTTTCCT 78

- (2) INFORMATION FOR SEQ ID NO:32:
 - (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 70 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

TTCTCGGTTG GTCTCTGGCG GAGCCAATCG GTGTACAATA TCTTCCTCTT 50
GTGTATGATT CGCTTTTCCC 70

- (2) INFORMATION FOR SEQ ID NO:33:
 - (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 72 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

TTCTCGGTTG GTCTCTGGCG GAGCCGTTAG CTGGTTAGTT AGTACTAGTC 50
TTGTGTATGA TTCGCTTTTC CC 72

- (2) INFORMATION FOR SEQ ID NO:34:
(i) SEQUENCE CHARACTERIZATION:
(A) LENGTH: 72 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULAR TYPE: DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

TTCTCGGTTG GTCTCTGGCG GAGCAGGTAA GCGATTATGG GGTATCCTC 50
TTGTGTATGA TTCGCTTTTC CC 72

- (2) INFORMATION FOR SEQ ID NO:35:
(i) SEQUENCE CHARACTERIZATION:
(A) LENGTH: 72 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULAR TYPE: DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

TTCTCGGTTG GTCTCTGGCG GAGCTAGTTA CATGAATAA TCGTGGAGTC 50
TTGTGTATGA TTCGCTTTTC CC 72

- (2) INFORMATION FOR SEQ ID NO:36:
(i) SEQUENCE CHARACTERIZATION:
(A) LENGTH: 78 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULAR TYPE: DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

TTCTCGGTTG GTCTCTGGCG GAGCGATGTA CAGTATCGCT ATCGAAAGAG 50
GCTGTCTTGT GTATGATTCG CTTTTCCC 78

- (2) INFORMATION FOR SEQ ID NO:37:
(i) SEQUENCE CHARACTERIZATION:
(A) LENGTH: 78 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULAR TYPE: DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

TTCTCGGTTG GTCTCTGGCG GAGCAGTGTG CAGTAGTGTG ATGTCAGAGT 50
ATCCTCTTGT GTATGATTCG CTTTTCCC 78

- (2) INFORMATION FOR SEQ ID NO:38:
(i) SEQUENCE CHARACTERIZATION:
(A) LENGTH: 78 base pairs
(B) TYPE: nucleic acid

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

TTCTCGGTTG GTCTCTGGCG GAGCAGTGTG CGGTAGTGTG ATCTGAGAGT 50
 ATCCTCTTGT GTATGATTCG CTTTCC 78

- (2) INFORMATION FOR SEQ ID NO:39:
 - (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 77 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

TTCTCGGTTG GTCTCTGGCG GAGCAGTGTG TAGTAGTGTT ACGATGGGGA 50
 CGGTCTTGTG TATGATTCG TTTCC 77

- (2) INFORMATION FOR SEQ ID NO:40:
 - (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 78 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

TTCTCGGTTG GTCTCTGGCG GAGCAGTGTG CAGTAGTGTT CCCGGTAGAG 50
 CTATTCTTGT GTATGATTCG CTTTCC 78

- (2) INFORMATION FOR SEQ ID NO:41:
 - (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 78 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

TTCTCGGTTG GTCTCTGGCG GAGCAATGTG CAGTATTGAT ATCGCTGGTG 50
 GTCATCTTGT GTATGATTCG CTTTCC 78

- (2) INFORMATION FOR SEQ ID NO:42:
 - (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 76 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

TTCTCGGTTG GTCTCTGGCG GAGCAAGTGT ACAGTAGTTG CCTACGCTAG 50
 TGTCTTGTGT ATGATTCGCT TTTCC 76

- (2) INFORMATION FOR SEQ ID NO:43:
- (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 78 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

TTCTCGGTTG GTCTCTGGCG GAGCAAGTGT GCAGTAGTTA CTCATAAGAG 50
ACCATCTTGT GTATGATTCG CTTTCC 78

- (2) INFORMATION FOR SEQ ID NO:44:
- (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 76 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

TTCTCGGTTG GTCTCTGGCG GAGCAAGTGT ACAGTAGTTG CCTACGCTAG 50
TGTCTTGTGT ATGATTCGCT TTTCC 76

- (2) INFORMATION FOR SEQ ID NO:45:
- (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 78 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

TTCTCGGTTG GTCTCTGGCG GAGCACAATG TGAAGTATTG GGGTACGTCA 50
GTAGTCTTGT GTATGATTCG CTTTCC 78

- (2) INFORMATION FOR SEQ ID NO:46:
- (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 78 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

TTCTCGGTTG GTCTCTGGCG GAGCCAAGCG GAAACAATGT ACAGTATTGG 50
GATCTCTTGT GTATGATTCG CTTTCC 78

- (2) INFORMATION FOR SEQ ID NO:47:
- (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 78 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

TTCTCGGTTG GTCTCTGGCG GAGCAAGGCC ATTGATGTAC AGTATCAATG 50
CTGCTCTTGT GTATGATTCG CTTTCC 78

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERIZATION:

- (A) LENGTH: 78 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

TTCTCGGTTG GTCTCTGGCG GAGCAATTGG GAAACAATGT GCAGTATGTG 50
AAGGTCTTGT GTATGATTCG CTTTCC 78

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERIZATION:

- (A) LENGTH: 78 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

TTCTCGGTTG GTCTCTGGCG GAGCAAATGG GAAACAATGT GCAGTATTGG 50
AAGGTCTTGT GTATGATTCG CTTTCC 78

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERIZATION:

- (A) LENGTH: 78 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

TTCTCGGTTG GTCTCTGGCG GAGCAAGACC AGACAATGTA CAGTATTGGC 50
CTGATCTTGT GTATGATTCG CTTTCC 78

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERIZATION:

- (A) LENGTH: 77 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

TTCTCGGTTG GTCTCTGGCG GAGCAGCTG ACAGGCCACG TTTGTGATG 50
ATTCTTGTGT ATGATTCGCT TTTCCCT 77

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERIZATION:

67

- (A) LENGTH: 78 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

TTCTCGGTTG GTCTCTGGCG GAGCGAGAAC TCCGTTCTTA GCGTATTGGA 50
GTCCTCTTGT GTATGATTCG CTTTTCCTCC 78

- (2) INFORMATION FOR SEQ ID NO:53:
 - (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 79 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

TTCTCGGTTG GTCTCTGGCG GAGCAGGTGG GACATTCTTT GCGTTATGTC 50
TCTGATCTTG TGTATGATTC GCTTTCCTCC 79

- (2) INFORMATION FOR SEQ ID NO:54:
 - (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 78 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

TTCTCGGTTG GTCTCTGGCG GAGCGGGCTC GGAACATTCT TAGCGTTTTG 50
TTCCTCTTGT GTATGATTCG CTTTTCCTCC 78

- (2) INFORMATION FOR SEQ ID NO:55:
 - (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 78 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

TTCTCGGTTG GTCTCTGGCG GAGCATAGGC AGGGGACATT GCAACCTTTT 50
GTCATCTTGT GTATGATTCG CTTTTCCTCC 78

- (2) INFORMATION FOR SEQ ID NO:56:
 - (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 78 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

TTCTCGGTTG GTCTCTGGCG GAGCAATTGA AGTGACTTTC TCTGCGTTTA 50
GTCGCTCTGT GTATGATTCG CTTTCC 78

- (2) INFORMATION FOR SEQ ID NO:57:
- (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 78 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

TTCTCGGTTG GTCTCTGGCG GAGCAGGAAT CTGGGGCATT CTTGCGTTT 50
TGGCTCTGT GTATGATTCG CTTTCC 78

- (2) INFORMATION FOR SEQ ID NO:58:
- (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 77 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

TTCTCGGTTG GTCTCTGGCG GAGCCTCAGG ATAAGGTCAT TCTAACGTTA 50
TGATCTGTG TATGATTCG TTTTCC 77

- (2) INFORMATION FOR SEQ ID NO:59:
- (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 78 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

TTCTCGGTTG GTCTCTGGCG GAGCGATCAT CTCAGAGCAT TCTTAGCGTT 50
TTGTTCTGT GTATGATTCG CTTTCC 78

- (2) INFORMATION FOR SEQ ID NO:60:
- (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 78 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

TTCTCGGTTG GTCTCTGGCG GAGCGATCAT CTAAGAGCAT TCTTAGCGTT 50
TTGGTCTGT GTATGATTCG CTTTCC 78

- (2) INFORMATION FOR SEQ ID NO:61:
- (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 78 base pairs
 - (B) TYPE: nucleic acid

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

TTCTCGGTTG GTCTCTGGCG GAGCCAAAAC GAGAGAGCTT TCTGTGCGTT 50
TAGCTCTTGT GTATGATTCTG CTTTTCCTC 78

- (2) INFORMATION FOR SEQ ID NO:62:
 - (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 78 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

TTCTCGGTTG GTCTCTGGCG GAGCGACCAA GCGTCAAGAT ATTCAAACGT 50
TTTATCTTGT GTATGATTCTG CTTTTCCTC 78

- (2) INFORMATION FOR SEQ ID NO:63:
 - (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 78 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

TTCTCGGTTG GTCTCTGGCG GAGCAGAAGC ATACGAAGAC ATTCCAACGT 50
TTGGTCTTGT GTATGATTCTG CTTTTCCTC 78

- (2) INFORMATION FOR SEQ ID NO:64:
 - (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 78 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

TTTCTCGGTT GGTCTCTGGC GGAGCAATCG ATTGTTGAAC ATTCTGACGT 50
TTTGTCTTGT GTATGATTCTG CTTTTCCTC 78

- (2) INFORMATION FOR SEQ ID NO:65:
 - (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 78 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

TTCTCGGTTG GTCTCTGGCG GAGCAGAAGC ATACGAAGAC ATTCCAACGT 50
TTTGTCTTGT GTATGATTCTG CTTTTCCTC 78

- (2) INFORMATION FOR SEQ ID NO:66:
- (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 78 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

TTCTCGGTTG GTCTCTGGCG GAGCAGAAGC ATACGAAGAC ATTCCAACGT 50
TTTGTCTTGT GTATGATTCTG CTTTTCCC 78

- (2) INFORMATION FOR SEQ ID NO:67:
- (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

GGGACCAGAC AATGTACAGT ATTGTCTGGT CCC 33

- (2) INFORMATION FOR SEQ ID NO:68:
- (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 27 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

GCCGGCCAAT GTACAGTATT GGCCGGC 27

- (2) INFORMATION FOR SEQ ID NO:69:
- (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

GGCCAATGTA CAGTATTGGC C 21

- (2) INFORMATION FOR SEQ ID NO:70:
- (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 51 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

TGGCGGAGCG ATCATCTCAG AGCATTCTTA GCGTTTTGTT CTTGTGTATG
A

50

51

(2) INFORMATION FOR SEQ ID NO:71:

(i) SEQUENCE CHARACTERIZATION:

- (A) LENGTH: 55 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: DNA

(ix) FEATURE:

- (D) OTHER INFORMATION: N at position 28 is
an inverted-orientation (3'-3' linkage)
phosphoramidite

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

GCCGGCCAAT GTACAGTATT GGCCGGCNCG GCCGGTTATG ACATGTAACC
GGCCG

50

55

(2) INFORMATION FOR SEQ ID NO:72:

(i) SEQUENCE CHARACTERIZATION:

- (A) LENGTH: 81 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

TGGCGGAGCG ATCATCTCAG AGCATTCTTA GCGTTTTGTT CTTGTGTATG
ATTGCGCGC CAATGTACAG TATTGGCCGG C

50

81

(2) INFORMATION FOR SEQ ID NO:73:

(i) SEQUENCE CHARACTERIZATION:

- (A) LENGTH: 81 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

GCCGGCCAAT GTACAGTATT GGCCGGCTTT TGGCGGAGCG ATCATCTCAG
AGCATTCTTA GCGTTTTGTT CTTGTGTATG A

50

81

(2) INFORMATION FOR SEQ ID NO:74:

(i) SEQUENCE CHARACTERIZATION:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:74:

AAGACCAGAG GCCAATGTAC AGTATTGGCC GCCTGA

36

(2) INFORMATION FOR SEQ ID NO:75:

- (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 97 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:75:

TTCGAGCGTG AATCTGAATT CGCGGCTAGC CAGCTTTTGC TGGCTAGCCG 50
CGGTGGGAAA CTGAGGTAGG TGTTTTCACC TACCTCAGTT TCCCACC 97

- (2) INFORMATION FOR SEQ ID NO:76:
 - (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

TCAATACACA AATTGATGTA CAGTGTGCGAT 30

- (2) INFORMATION FOR SEQ ID NO:77:
 - (i) SEQUENCE CHARACTERIZATION:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULAR TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:77:

CATTGGGCCA GAGGAACACA ACCTCAACAG 30

CLAIMS:

1. A method of identifying nucleic acid ligands to a polymerase, comprising:
 - a) preparing a candidate mixture of nucleic acids;
 - 5 b) contacting the candidate mixture of nucleic acids with said polymerase, wherein nucleic acids having an increased affinity to the polymerase relative to the candidate mixture may be partitioned from the remainder of the candidate mixture;
 - c) partitioning the increased affinity nucleic acids from the
10 remainder of the candidate mixture; and
 - d) amplifying the increased affinity nucleic acids to yield a mixture of nucleic acids enriched for nucleic acid sequences with relatively higher affinity and specificity for binding to the polymerase, whereby nucleic acid ligands of the polymerase may be identified.
- 15 2. The method of claim 1 further comprising:
 - e) repeating steps b), c), and d).
3. The method of claim 1 wherein said polymerase is a DNA
20 polymerase.
4. The method of claim 1 wherein said polymerase is a reverse transcriptase.
- 25 5. The method of claim 1 wherein said polymerase is thermostable.
6. The method of claim 3 wherein said DNA polymerase is isolated from *Thermus aquaticus* (*Taq* polymerase).

7. The method of claims 3 or 4 wherein said polymerase is isolated from *Thermus thermophilus* (*Tth* polymerase).

8. The method of claim 1 wherein said candidate mixture of nucleic acids is comprised of single stranded nucleic acids.

9. The method of claim 1 wherein said single stranded nucleic acids are deoxyribonucleic acids.

10. A method for inhibiting the activity of a DNA polymerase, comprising adding an effective amount of a high affinity DNA polymerase nucleic acid ligand to a DNA polymerization reaction which is being maintained at ambient temperatures.

11. The method of claim 10 wherein said DNA polymerase nucleic acid ligand is identified according to the method of claim 1.

12. The method of claim 10 wherein said DNA polymerase is *Taq* polymerase.

13. The method of claim 12 wherein said polymerase ligand is a DNA selected from one of the ligands of Table 2 (SEQ ID NOS:7-35), Table 3 (SEQ ID NOS:36-66, 76, 77), Table 4 (SEQ ID NOS:67-73) or Table 5 (SEQ ID NO:74).

14. The method of claim 10 wherein said DNA polymerase is *Tth* polymerase.

15. The method of claim 14 wherein said polymerase ligand is a DNA

selected from one of the ligands of Table 2 (SEQ ID NOS:7-35), Table 3 (SEQ ID NOS:36-66, 76, 77), Table 4 (SEQ ID NOS:67-73) or Table 5 (SEQ ID NO:74).

5 16. A purified and isolated non-naturally occurring nucleic acid ligand to a polymerase.

 17. The nucleic acid ligand of claim 16 identified according to the method of claim 1.

10

 18. The purified and isolated non-naturally occurring nucleic acid ligand of claim 16, wherein said polymerase is *Taq* polymerase and wherein said ligand is selected from the group consisting of the sequences set forth in Table 2 (SEQ ID NOS:7-35), Table 3 (SEQ ID NOS:36-66, 76, 77), Table 4 (SEQ ID
15 NOS:67-73) or Table 5 (SEQ ID NO:74), or the corresponding complementary sequences thereof.

 19. The purified and isolated non-naturally occurring nucleic acid ligand of claim 16, wherein said polymerase is *Taq* polymerase, and wherein said
20 ligand is a DNA substantially homologous to and has substantially the same ability to bind *Taq* polymerase as a ligand selected from the group consisting of the sequences set forth in Table 2 (SEQ ID NOS:7-35), Table 3 (SEQ ID NOS:36-66, 76, 77), Table 4 (SEQ ID NOS:67-73) or Table 5 (SEQ ID NO:74), or the corresponding complementary sequences thereof.

25

 20. The purified and isolated non-naturally occurring nucleic acid ligand of claim 16, wherein said polymerase is *Taq* polymerase, and wherein said nucleic acid ligand has substantially the same structure and substantially the same ability to bind *Taq* polymerase as a ligand selected from the group consisting of

the sequences set forth in Table 2 (SEQ ID NOS:7-35), Table 3 (SEQ ID NOS:36-66, 76, 77), Table 4 (SEQ ID NOS:67-73) or Table 5 (SEQ ID NO:74), or the corresponding complementary sequences thereof.

5 21. The purified and isolated non-naturally occurring nucleic acid ligand of claim 16, wherein said polymerase is *Tth* polymerase, and wherein said nucleic acid ligand is selected from the group consisting of the sequences set forth in Table 2 (SEQ ID NOS:7-35), Table 3 (SEQ ID NOS:36-66, 76, 77), Table 4 (SEQ ID NOS:67-73) or Table 5 (SEQ ID NO:74), or the corresponding
10 complementary sequences thereof.

 22. The purified and isolated non-naturally occurring nucleic acid ligand of claim 16, wherein said polymerase is *Tth* polymerase, and wherein said nucleic acid ligand is substantially homologous to and has substantially the same
15 ability to bind *Tth* polymerase as a ligand selected from the group consisting of the sequences set forth in Table 2 (SEQ ID NOS:7-35), Table 3 (SEQ ID NOS:36-66, 76, 77), Table 4 (SEQ ID NOS:67-73) or Table 5 (SEQ ID NO:74), or the corresponding complementary sequences thereof.

20 23. The purified and isolated non-naturally occurring nucleic acid ligand of claim 16, wherein said polymerase is *Tth* polymerase, and wherein said nucleic acid ligand has substantially the same structure and substantially the same ability to bind *Tth* polymerase as a ligand selected from the group consisting of the sequences set forth in Table 2 (SEQ ID NOS:7-35), Table 3 (SEQ ID
25 NOS:36-66, 76, 77), Table 4 (SEQ ID NOS:67-73) or Table 5 (SEQ ID NO:74), or the corresponding complementary sequences thereof.

 24. A purified and isolated non-naturally occurring nucleic acid ligand to a reverse transcriptase.

25. The nucleic acid ligand to the reverse transcriptase of claim 24 identified according to the method comprising:

- a) preparing a candidate mixture of nucleic acids;
- b) contacting the candidate mixture of nucleic acids with the
5 reverse transcriptase, wherein nucleic acids having an increased affinity to the reverse transcriptase relative to the candidate mixture may be partitioned from the remainder of the candidate mixture;
- c) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture; and
- 10 d) amplifying the increased affinity nucleic acids to yield a mixture of nucleic acids enriched for nucleic acid sequences with relatively higher affinity and specificity for binding to the reverse transcriptase, whereby nucleic acid ligands of the reverse transcriptase may be identified.

15 26. The purified and isolated non-naturally occurring nucleic acid ligand of claim 24, wherein said reverse transcriptase is *Tth* polymerase, and wherein said nucleic acid ligand is selected from the group consisting of the sequences set forth in Table 2 (SEQ ID NOS:7-35), Table 3 (SEQ ID NOS:36-66, 76, 77), Table 4 (SEQ ID NOS:67-73) or Table 5 (SEQ ID NO:74), or the
20 corresponding complementary sequences thereof.

27. The purified and isolated non-naturally occurring nucleic acid ligand of claim 24, wherein said reverse transcriptase is *Tth* polymerase, and wherein said nucleic acid ligand is substantially homologous to and has
25 substantially the same ability to bind *Tth* polymerase as a ligand selected from the group consisting of the sequences set forth in Table 2 (SEQ ID NOS:7-35), Table 3 (SEQ ID NOS:36-66, 76, 77), Table 4 (SEQ ID NOS:67-73) or Table 5 (SEQ ID NO:74), or the corresponding complementary sequences thereof.

28. The purified and isolated non-naturally occurring nucleic acid ligand of claim 24, wherein said reverse transcriptase is *Tth* polymerase, and wherein said nucleic acid ligand has substantially the same structure and substantially the same ability to bind *Tth* polymerase as a ligand selected from the group consisting of the sequences set forth in Table 2 (SEQ ID NOS:7-35), Table 3 (SEQ ID NOS:36-66, 76, 77), Table 4 (SEQ ID NOS:67-73) or Table 5 (SEQ ID NO:74), or the corresponding complementary sequences thereof.

29. A method for performing the Polymerase Chain Reaction (PCR) comprising:

a) mixing a sample containing a nucleic acid sequence that is to be amplified with primers that are complementary to the sequences that flank the sequence to be amplified, a thermostable polymerase, and a nucleic acid ligand that is capable of inhibiting the polymerase at ambient temperatures, yet allows the polymerase to be activated at elevated temperatures; and

b) performing the standard PCR steps of melting the target nucleic acid, annealing the primers to the target nucleic acid, and synthesizing the target nucleic acid, by thermal cycling of the mixture.

30. The method of claim 29 wherein said thermostable polymerase is *Taq* polymerase and wherein said nucleic acid ligand is selected from the group consisting of the sequences set forth in Table 2 (SEQ ID NOS:7-35), Table 3 (SEQ ID NOS:36-66, 76, 77), Table 4 (SEQ ID NOS:67-73) or Table 5 (SEQ ID NO:74), or the corresponding complementary sequences thereof.

31. The method of claim 29 wherein said thermostable polymerase is *Taq* polymerase and wherein said nucleic acid ligand substantially homologous to and has substantially the same ability to bind *Taq* polymerase as a ligand selected from the group consisting of the sequences set forth in Table 2 (SEQ ID

NOS:7-35), Table 3 (SEQ ID NOS:36-66, 76, 77), Table 4 (SEQ ID NOS:67-73) or Table 5 (SEQ ID NO:74), or the corresponding complementary sequences thereof.

5 32. The method of claim 29 wherein said thermostable polymerase is *Taq* polymerase and wherein said nucleic acid ligand has substantially the same structure and substantially the same ability to bind *Taq* polymerase as a ligand selected from the group consisting of the sequences set forth in Table 2 (SEQ ID NOS:7-35), Table 3 (SEQ ID NOS:36-66, 76, 77), Table 4 (SEQ ID NOS:67-73)
10 or Table 5 (SEQ ID NO:74), or the corresponding complementary sequences thereof.

 33. The method of claim 29 wherein said thermostable polymerase is *Tth* polymerase, and wherein said nucleic acid ligand is selected from the group consisting of the sequences set forth in Table 2 (SEQ ID NOS:7-35), Table 3
15 (SEQ ID NOS:36-66, 76, 77), Table 4 (SEQ ID NOS:67-73) or Table 5 (SEQ ID NO:74), or the corresponding complementary sequences thereof.

 34. The method of claim 29 wherein said thermostable polymerase is
20 *Tth* polymerase, and wherein said nucleic acid ligand is substantially homologous to and has substantially the same ability to bind *Tth* polymerase as a ligand selected from the group consisting of the sequences set forth in Table 2 (SEQ ID NOS:7-35), Table 3 (SEQ ID NOS:36-66, 76, 77), Table 4 (SEQ ID NOS:67-73) or Table 5 (SEQ ID NO:74), or the corresponding complementary sequences
25 thereof.

 35. The method of claim 29 wherein said thermostable polymerase is *Tth* polymerase, and wherein said nucleic acid ligand has substantially the same structure and substantially the same ability to bind *Tth* polymerase as a ligand

selected from the group consisting of the sequences set forth in Table 2 (SEQ ID NOS:7-35), Table 3 (SEQ ID NOS:36-66, 76, 77), Table 4 (SEQ ID NOS:67-73) or Table 5 (SEQ ID NO:74), or the corresponding complementary sequences thereof.

5

36. A PCR kit comprising a thermostable DNA polymerase and a nucleic acid ligand that inhibits said polymerase at ambient temperatures, yet allows the polymerase to be activated at elevated temperatures.

10

37. The PCR kit of claim 36 wherein said thermostable DNA polymerase is *Taq* polymerase and wherein said nucleic acid ligand is selected from the group consisting of the sequences set forth in Table 2 (SEQ ID NOS:7-35), Table 3 (SEQ ID NOS:36-66, 76, 77), Table 4 (SEQ ID NOS:67-73) or Table 5 (SEQ ID NO:74), or the corresponding complementary sequences thereof.

15

38. The PCR kit of claim 36 wherein said thermostable DNA polymerase is *Tth* polymerase and wherein said nucleic acid ligand is selected from the group consisting of the sequences set forth in Table 2 (SEQ ID NOS:7-35), Table 3 (SEQ ID NOS:36-66, 76, 77), Table 4 (SEQ ID NOS:67-73) or Table 5 (SEQ ID NO:74), or the corresponding complementary sequences thereof.

20

39. An improved method for performing the Polymerase Chain Reaction (PCR), wherein said improvement comprises the step of adding to the thermostable polymerase a nucleic acid ligand that inhibits said polymerase at ambient temperatures, yet allows the polymerase to be activated at the elevated temperature cycles of the PCR process.

25

40. A method of identifying a nucleic acid switch, comprising:

a) preparing a candidate mixture of nucleic acids;

b) contacting the candidate mixture of nucleic acids with a target compound, wherein nucleic acids having an increased affinity to the target relative to the candidate mixture may be partitioned from the remainder of the candidate mixture;

5 c) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture conditions, whereby increased affinity nucleic acids are further partitioned based on their lack of affinity to the target upon the variation of an environmental parameter; and

10 d) amplifying the increased affinity nucleic acids to yield a mixture of nucleic acids enriched for nucleic acid sequences with relatively higher affinity and specificity for binding to the polymerase, whereby nucleic acid ligands of the polymerase may be identified.

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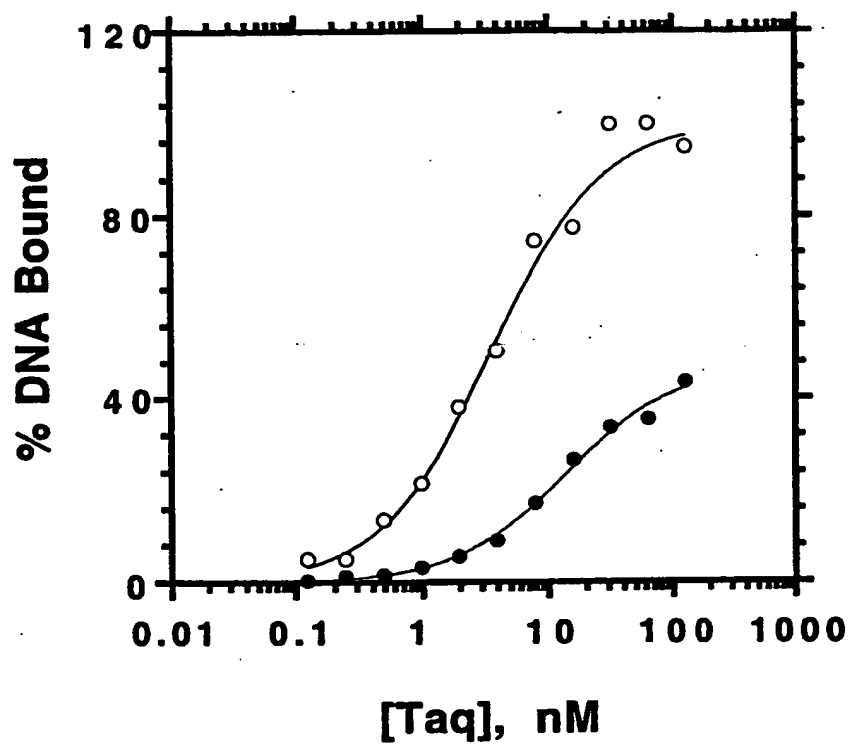


Fig. 1A

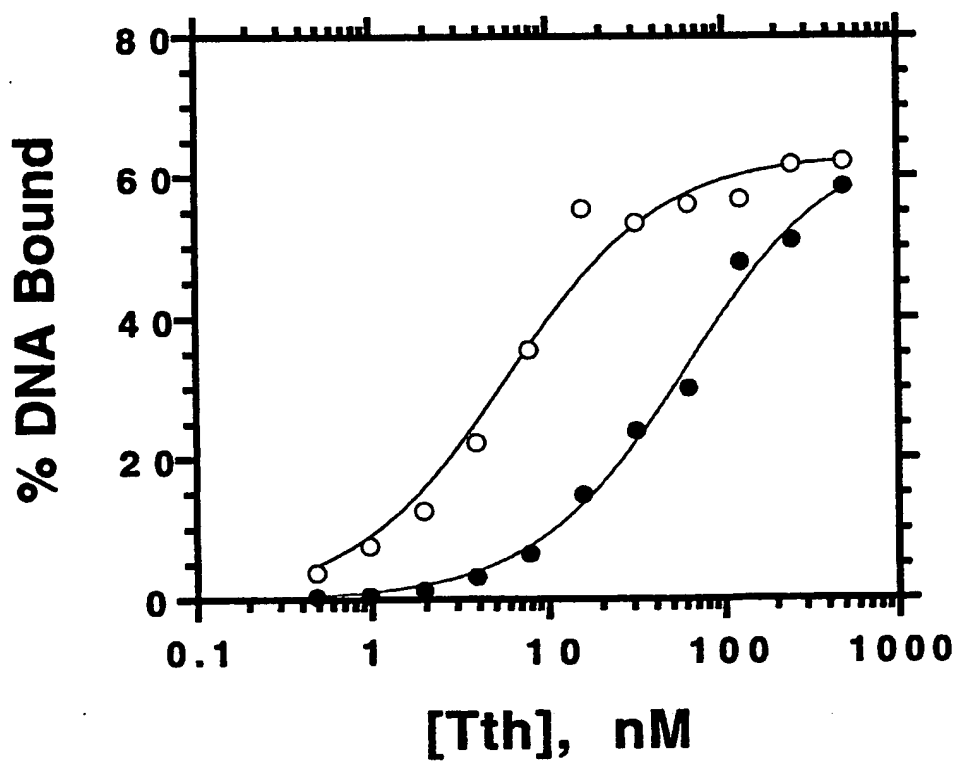


Fig. 1B

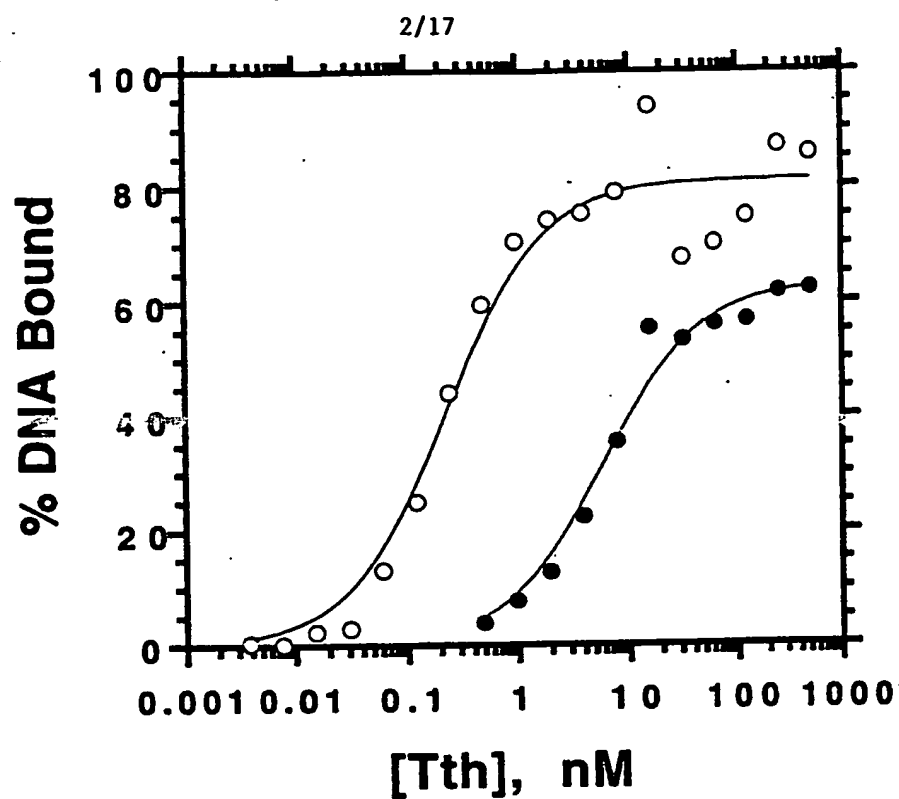


Fig. 2A

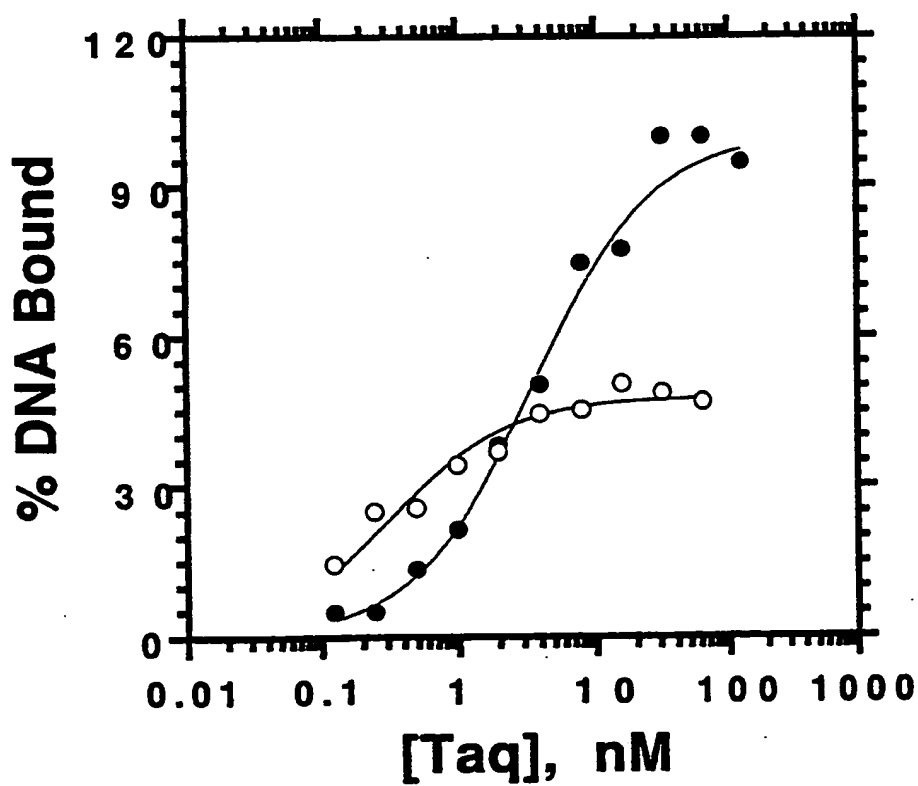


Fig. 2B

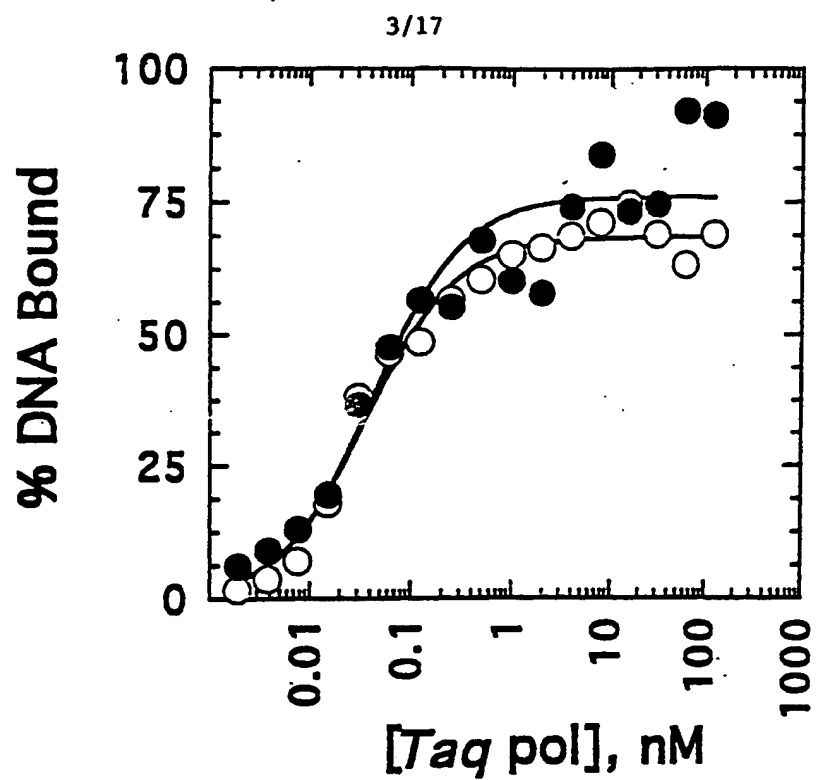


Fig. 3A

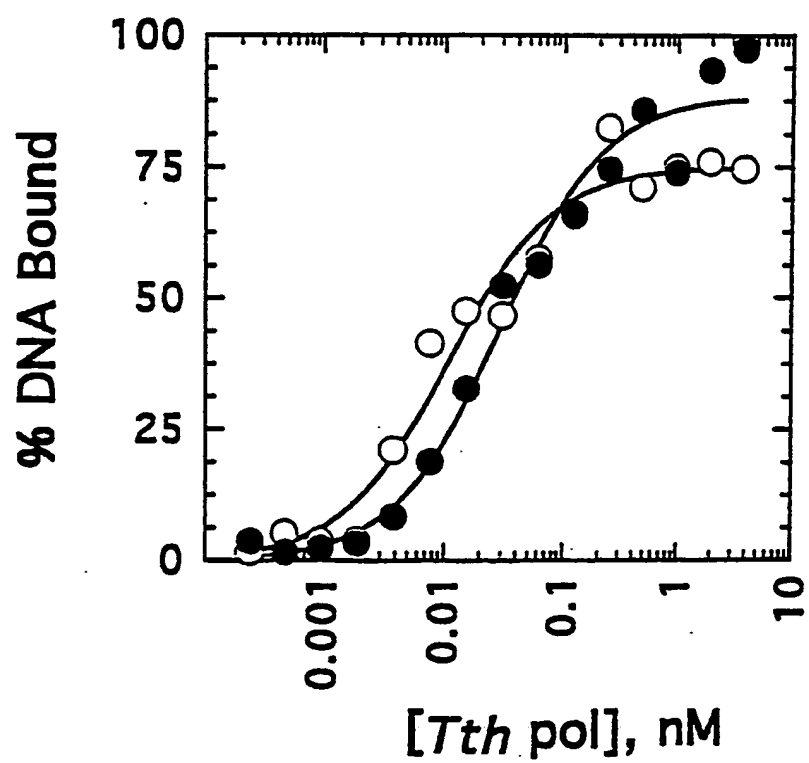


Fig. 3B

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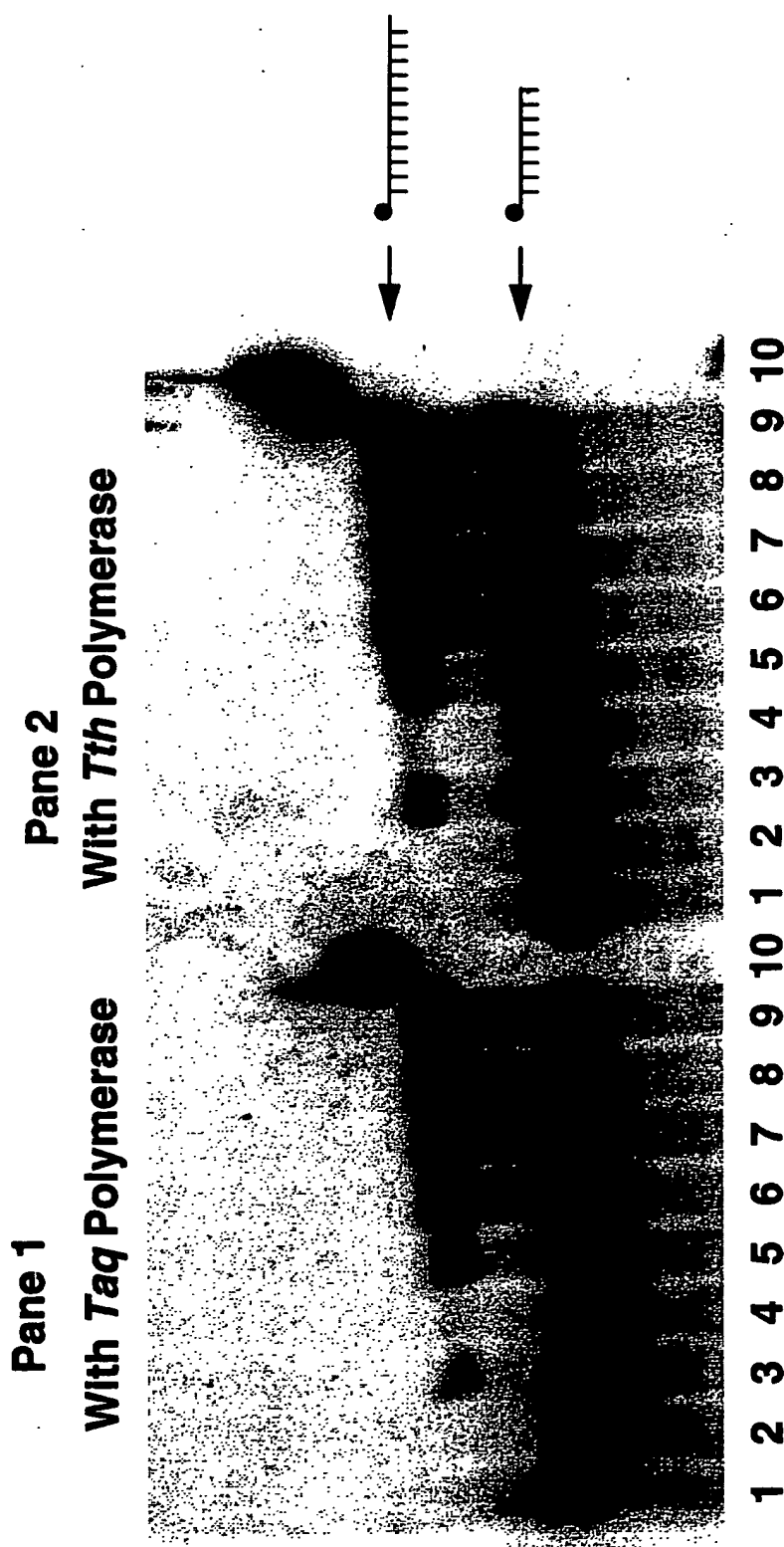


FIGURE 5A

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FIGURE 5B

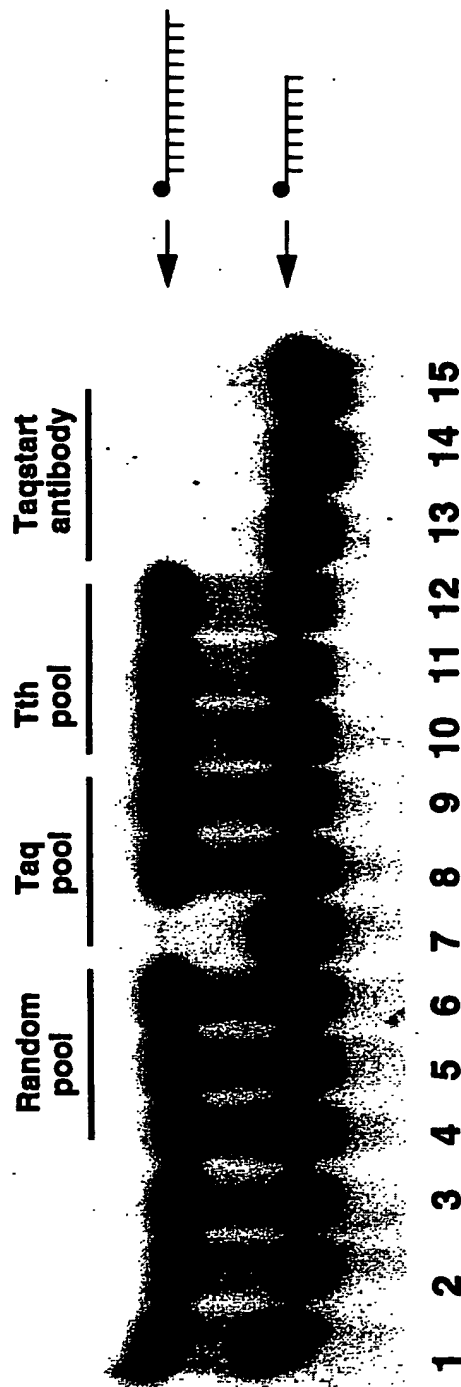
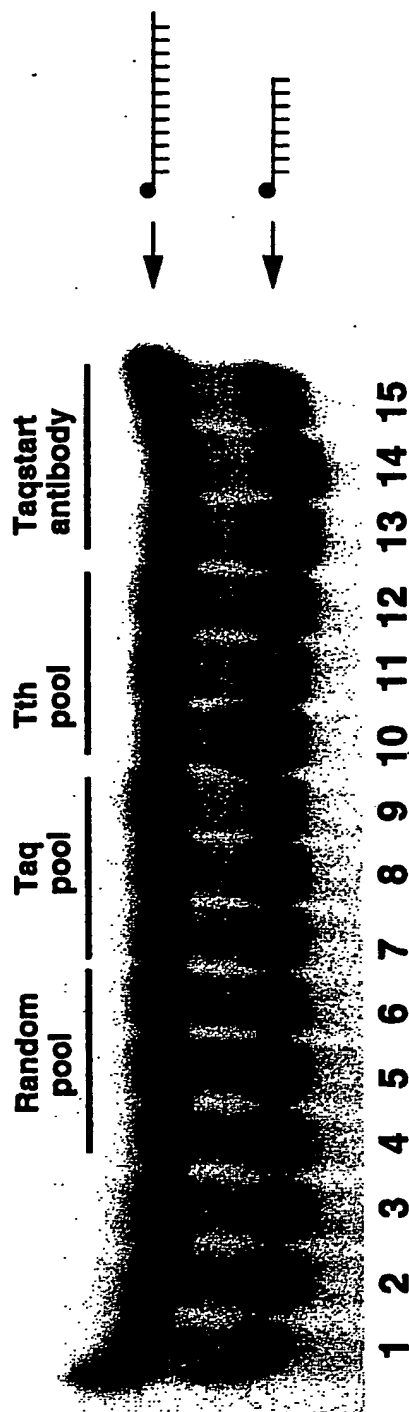
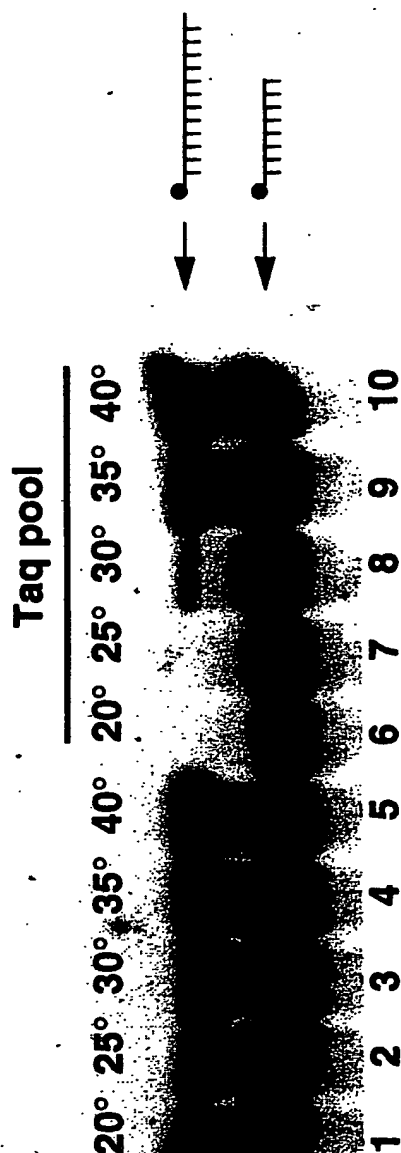
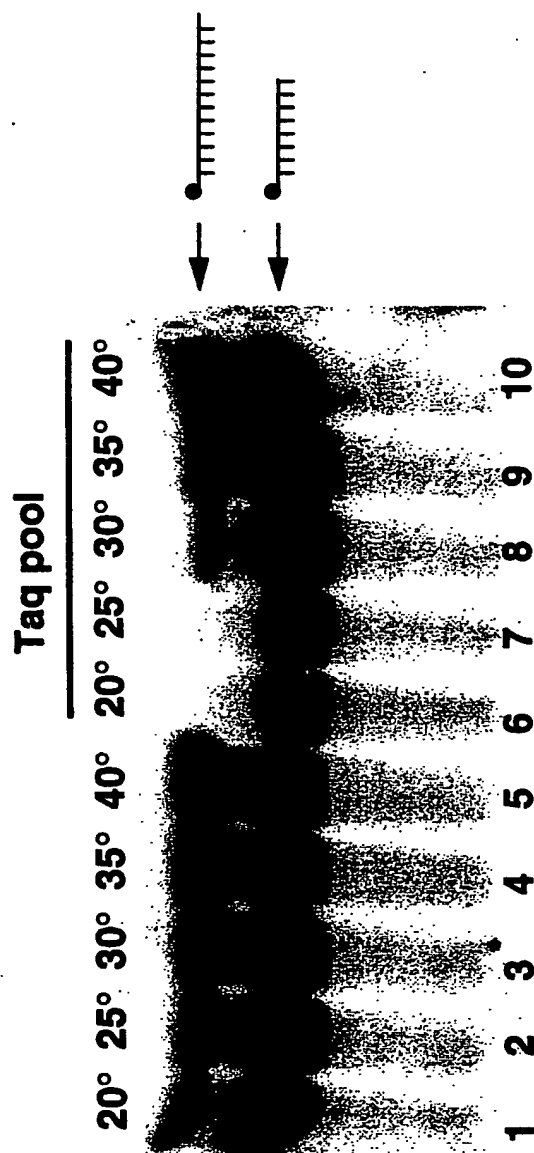


FIGURE 5C





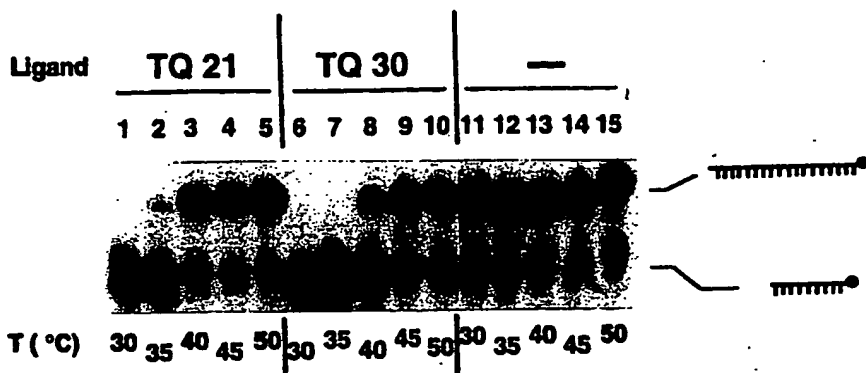
Taq pol

Fig. 6A

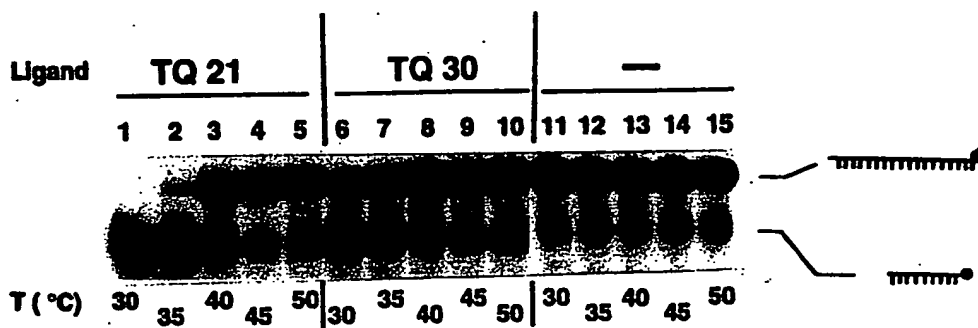
Tth pol

Fig. 6B

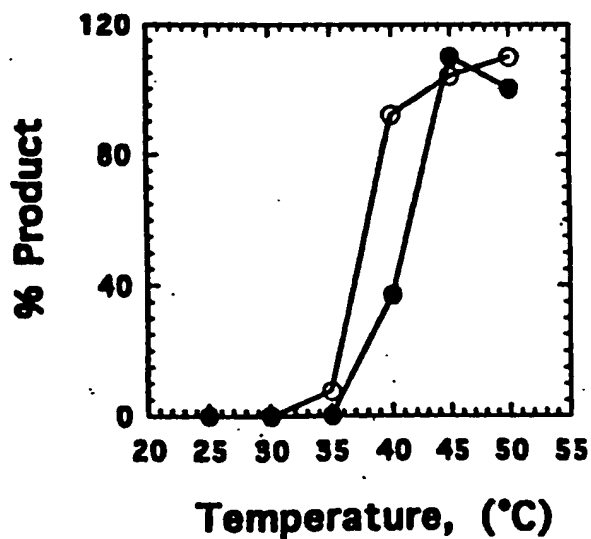


Fig. 6C

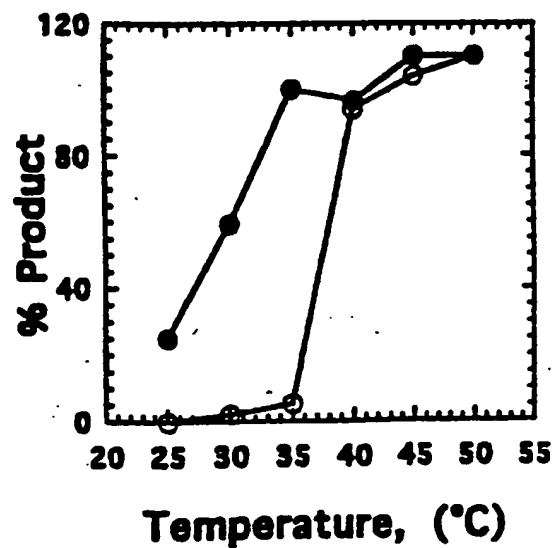
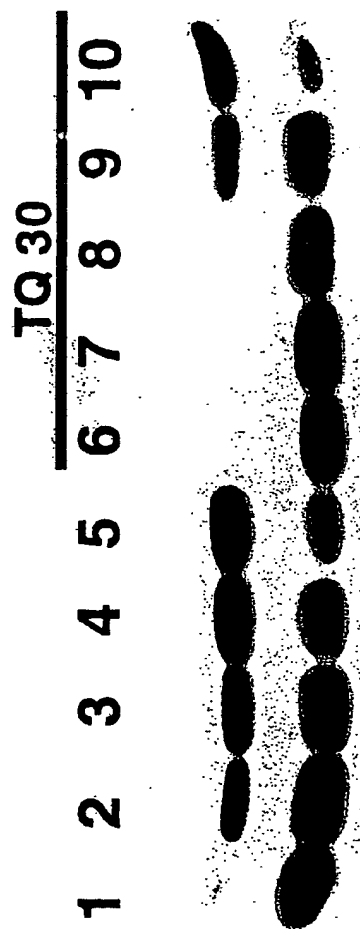


Fig. 6D



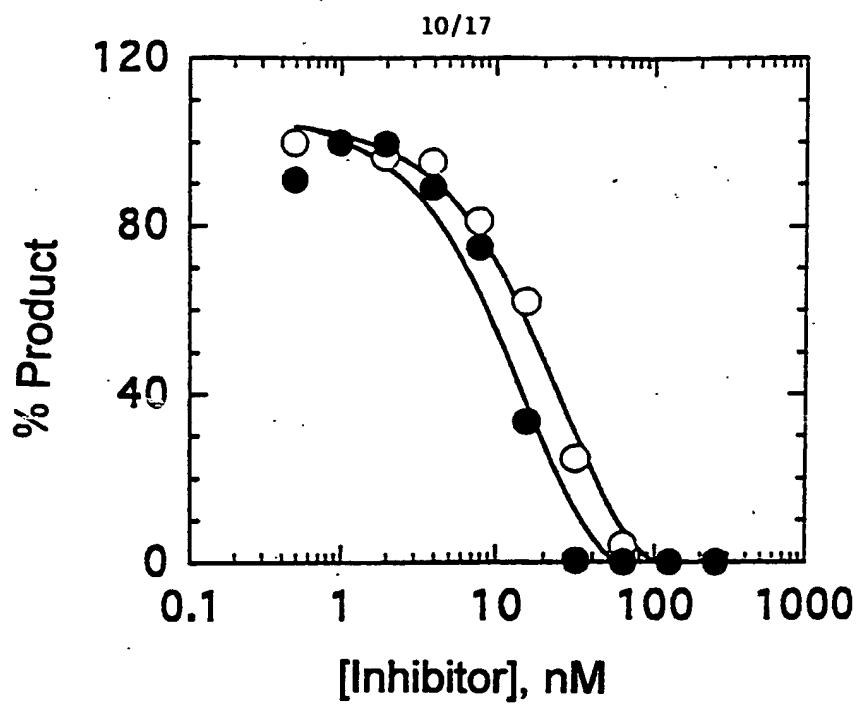


Fig. 8A

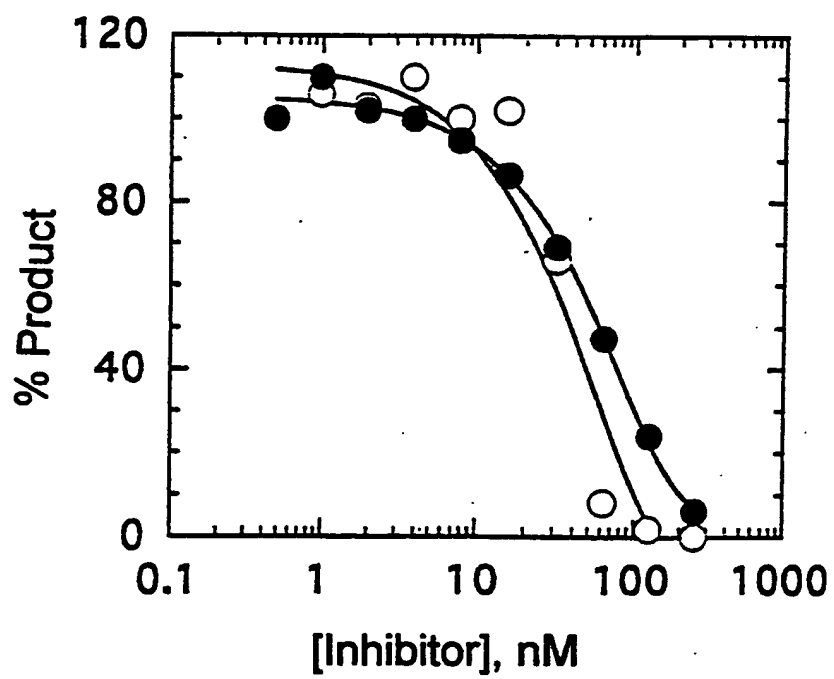


Fig. 8B

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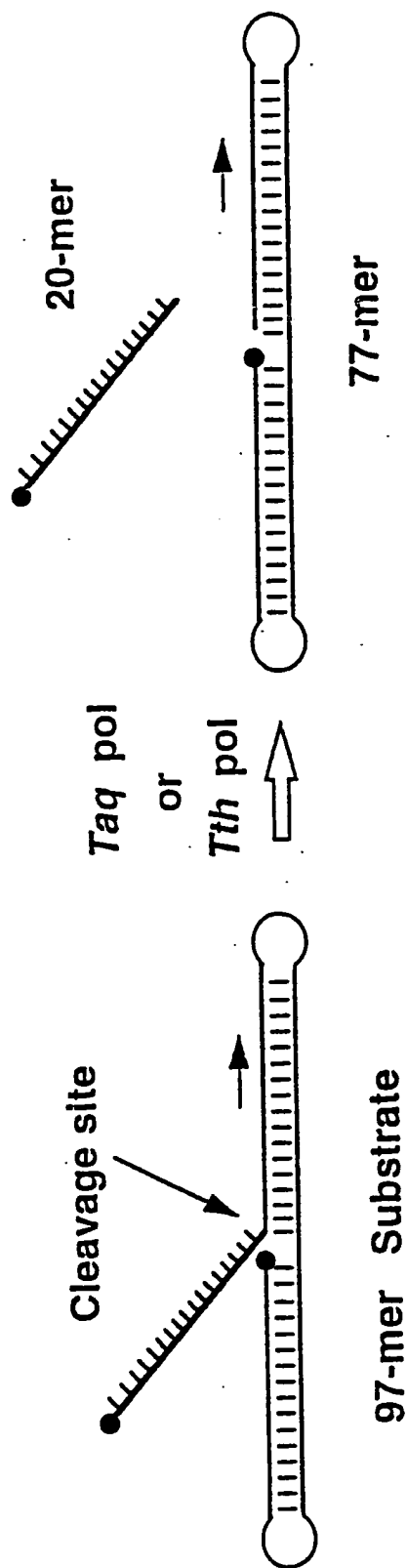


Fig. 9

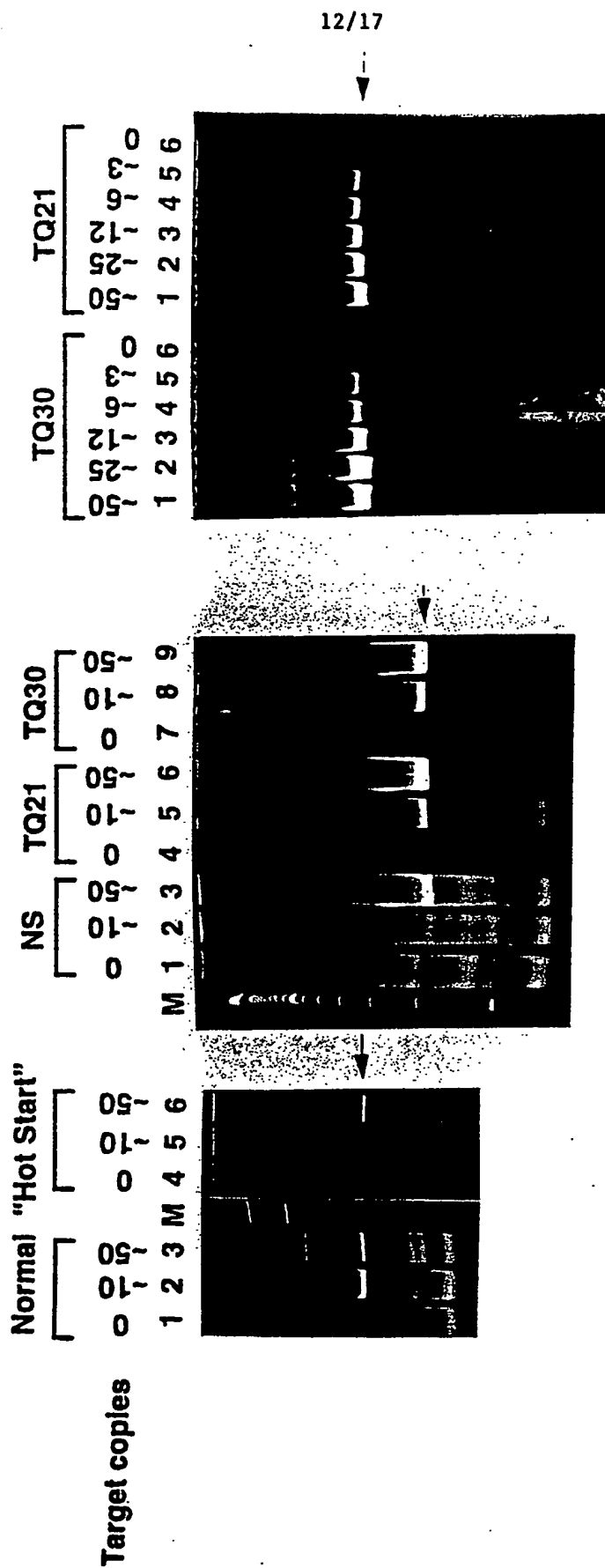


Fig. 10C

Fig. 10B

Fig. 10A

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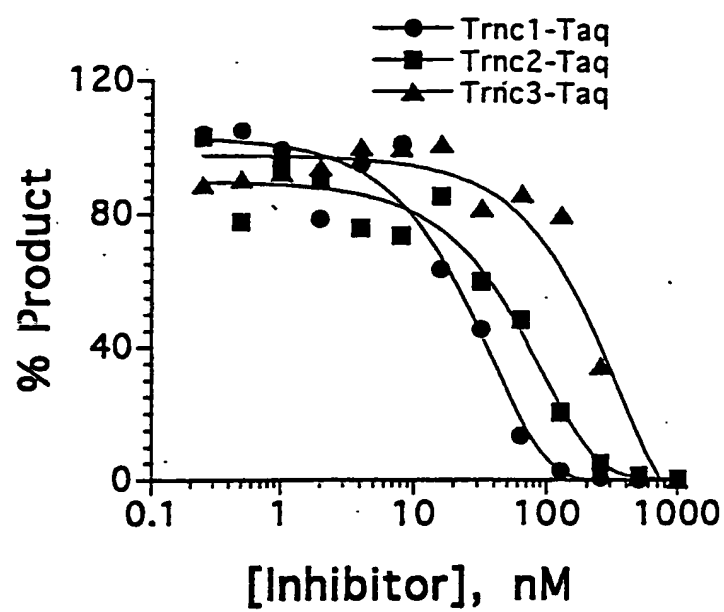


Fig. 11

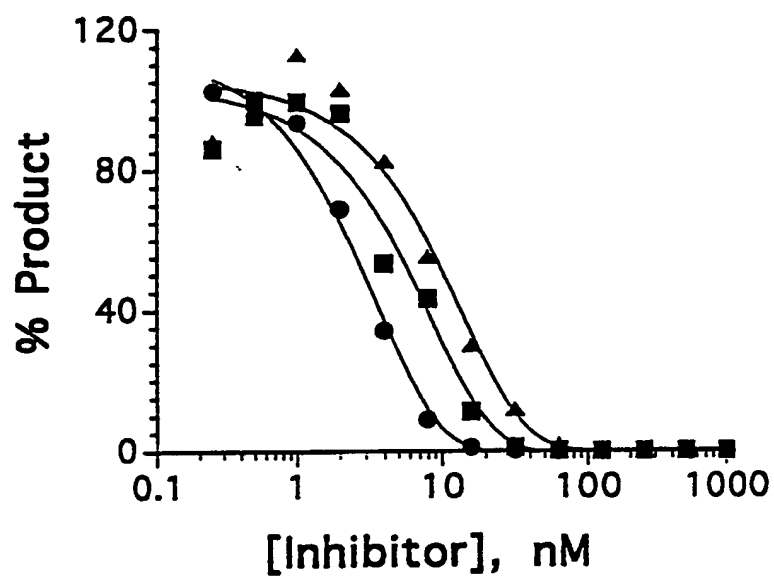


Fig. 12

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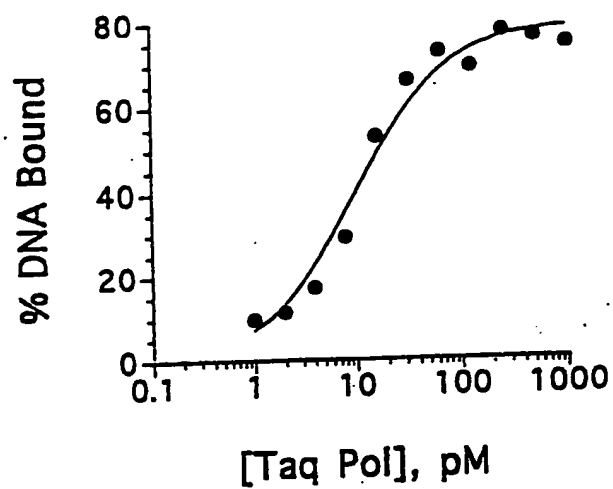


Fig. 13A

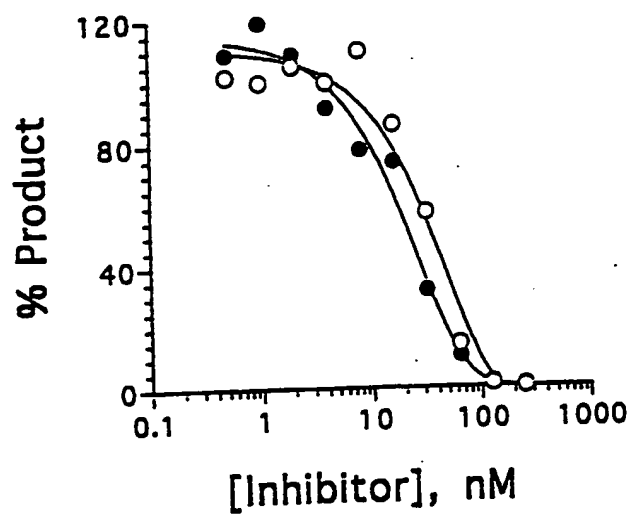


Fig. 13B

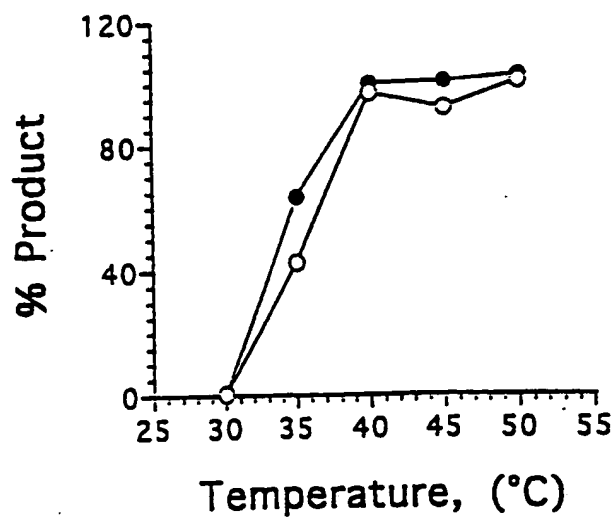


Fig. 13C

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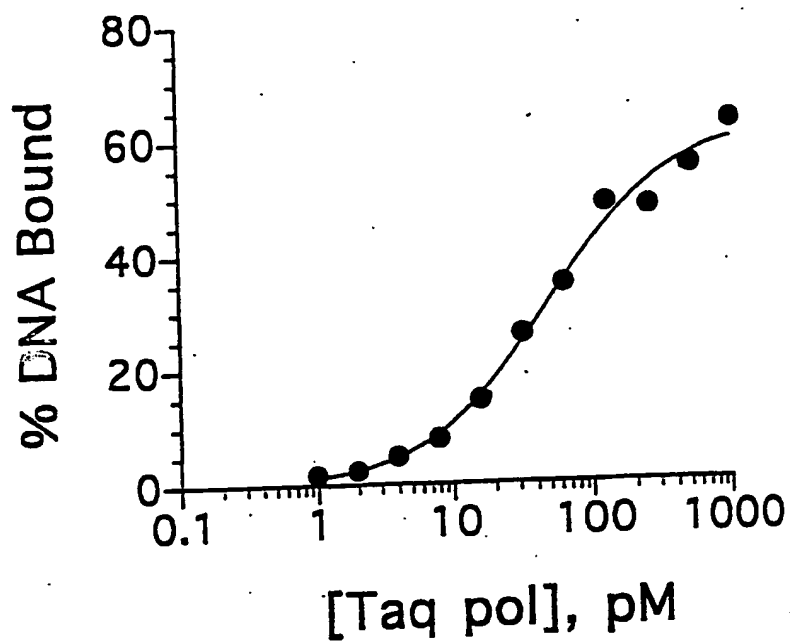


Fig. 14A

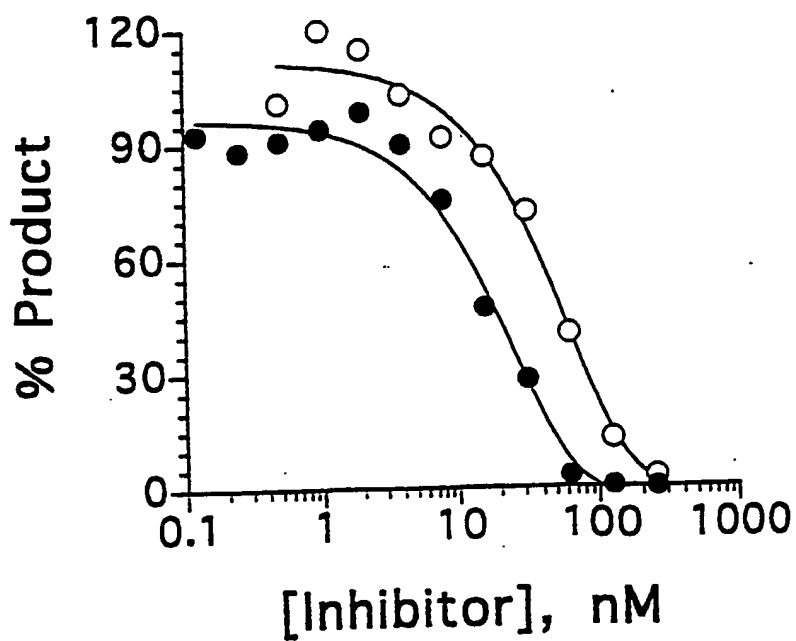


Fig. 14B

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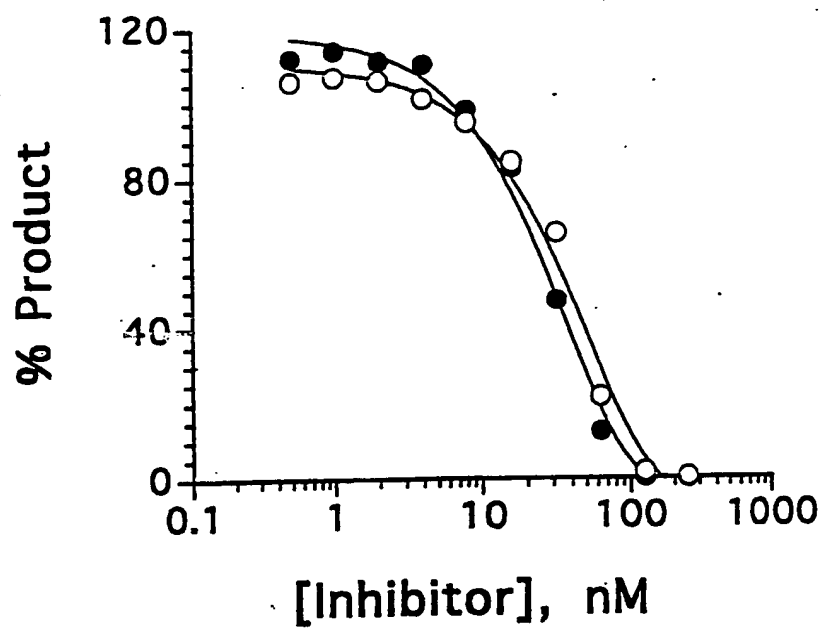


Fig. 15A

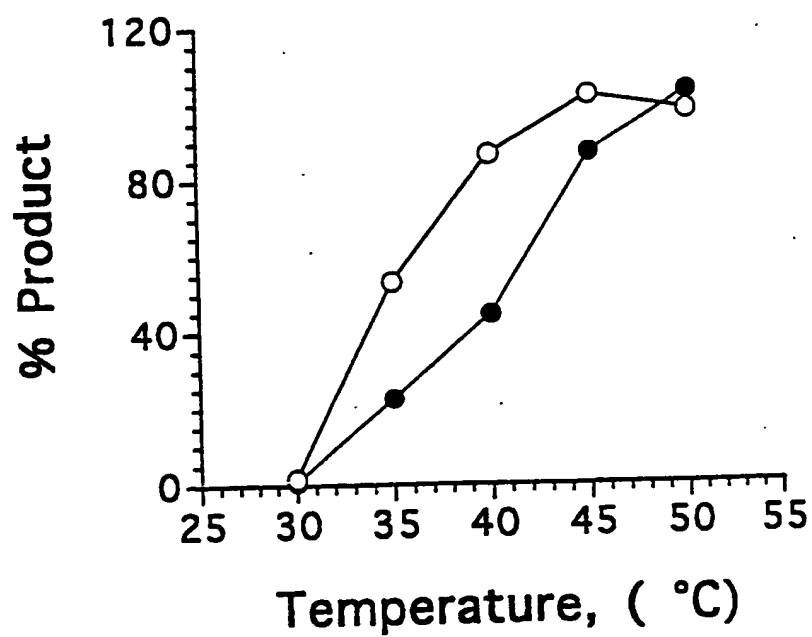


Fig. 15B

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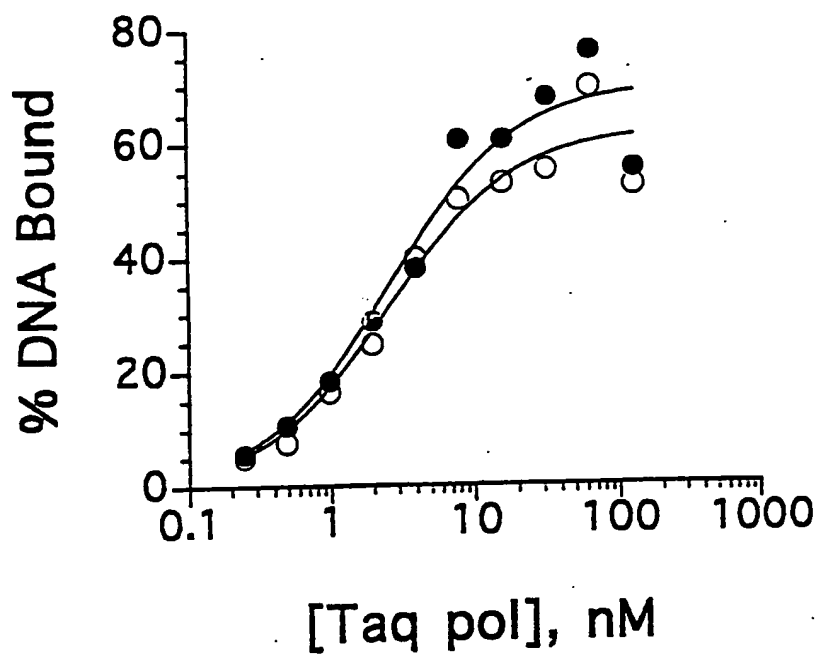


Fig. 16A

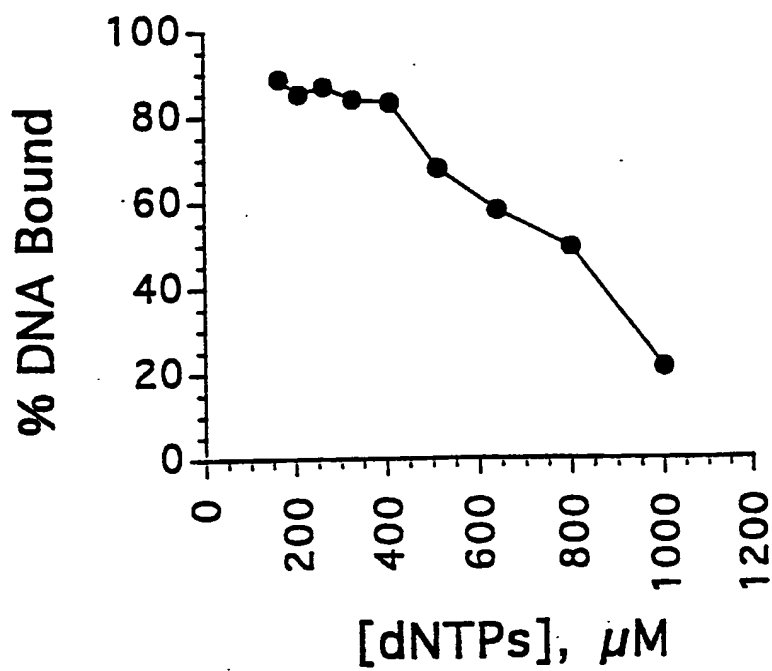


Fig. 16B

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/09451

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/68; C12P 19/34; C07H 21/04

US CL :435/6, 91.2; 536/24.3

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.2; 536/24.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAPLUS, BIOSIS, MEDLINE, EMBASE, DRUGU

search terms:

SELEX, polymerase, reverse transcriptase, inhibitors

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P — Y,P	US 5,503,978 A (SCHNEIDER ET AL.) 02 April 1996, columns 6 and 14.	1-4, 8-11, 16, 17, 24, 25 — 5-7, 12-15, 18-23, 26-39
X — Y	CHEN et al. Selection of High-Affinity RNA Ligands to Reverse Transcriptase: Inhibition of cDNA Synthesis and RNase H Activity. Biochemistry. 1994, Vol. 33, pages 8746-8756. See pages 8748-8751.	1-4, 8-11, 16, 17, 24, 25 — 5-7, 12-15, 18-23, 26-39
Y	US 5,338,671 A (SCALICE ET AL.) 16 August 1994. See columns 3-4 and 10.	29-39

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reasons (as specified)	"Z" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

07 AUGUST 1996

Date of mailing of the international search report

02 OCT 1996

Name and mailing address of the ISA/US
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/09451

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KELLOGG et al. TaqStart Antibody: "Hot Start" PCR Facilitated by a Neutralizing Monoclonal Antibody Directed Against Taq DNA Polymerase. BioTechniques. 1994, Vol. 16, No. 6, pages 1134-1137. See pages 1135-1136.	29-39
X	TUERK et al. RNA pseudoknots that inhibit human immunodeficiency virus type 1 reverse transcriptase. Proc. Natl. Acad. Sci. USA. August 1992. Vol. 89, pages 6988-6992. See page 6991, column 2.	1-4, 8, 10, 11, 16, 17, 24, 25
Y		5-7, 9, 29, 39

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/09451

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-39

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/09451

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-39, drawn to a first process of making nucleic acid ligands to polymerase; a first product, nucleic acid ligand to polymerase; and a first process and kit of using the nucleic acid ligands to inhibit polymerase activity.

Group II, claim 40, drawn to a second process of making nucleic acid ligands.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The second process of making, the Group II invention, has an additional partitioning step over the first process of making in Group I, wherein the additional partitioning step is based on lack of affinity of ligands to the target under different conditions from those of the first partitioning step. Note that PCT Rule 13 does not provide for multiple methods of making (see 37 CFR 1.475(d)). Therefore, the additional partitioning step does not constitute a special technical feature as defined by PCT Rule 13.2.

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